UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

CRYPTOSPORIDIOSIS IN PRE-WEANED CALVES – OBSERVATIONS IN GERMANY AND PORTUGAL

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DISSERTAÇÃO DE MESTRADO EM MEDICINA VETERINÁRIA

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Acknowledgements:

At this final step and facing the end of an important period of my life I would like to thank some important people that made this possible.

First of all I want to thank my family for always standing by my side, supporting my decisions and helping me in every step. Thank you for all your love, understanding, encouragement and positivity.

I want to thank all my friends that I met throughout this wonderful period. To all the special people I met in Portugal, Hungary and Germany, thank you, obrigada, köszönöm, danke! for your support, help, friendship, fun, smiles, travels and fantastic moments.

For the realization of this dissertation, my thanks go to my supervisors Prof. Dr. Axel Wehrend and Dr. Marlene Sickinger for the introduction to the german reality in bovine medicine and the increase of my interest towards this field. Thank you for your support, knowledge, patience and rigor.

To my supervisor in Lisbon, Prof. Dr. George Stilwell, for the help, patience, encouragement, knowledge and disposal I owe gratitude.

I want to thank all the vets and employees from the Clinics for Ruminants – Internal Medicine and Surgery (Klinik für Wiederkäuer – Innere Medizin und Chirurgie) and from the Clinics for Obstetrics, Gynecology and Andrology with veterinary ambulance (Klinik für Geburtshilfe, Gynäkologie und Andrologie der Gross- und Kleintiere mit Tierärztlicher Ambulanz) from the Justus Liebig University Giessen, Germany. Thank you for your teaching, patience, sympathy and nice moments.

Sincere thanks also go to everybody from the veterinary clinic Dr. Karsten von Brehm in Niesgrau. Thank you for the introduction to real farm work, support, friendship and for the teaching.

For the gathering of information and academical help, I want to thank Prof. Dr. Miguel Saraiva Lima, Prof. Dr. Isabel Fonseca, Carla Mendonça, Joana Gonçalves, Inês Grenho Ajuda, Teresa Duarte, Lídia Nabais.

And last but not least, a very special thank you goes to the cows, sheep, goats, horses, pigs, dogs, cats, rabbits and all the other animals that give all this work a meaning.

Resumo: Criptosporidiose em vitelos pré-desmame – Observações na Alemanha e em Portugal

A criptosporidiose é uma das mais importantes causas de diarreia neonatal bovina, sendo uma potencial ameaça à produção e uma importante causa de prejuízos económicos.

Vitelos recém-nascidos e até ao desmame são especialmente susceptíveis à infecção e desenvolvimento de sinais clínicos pelo parasita protozoário *Cryptosporidium parvum*, já que nascem quase completamente desprovidos de mecanismos de imunidade naturais.

Este parasita é considerado existente mundialmente, ubiquitário, sem especificidade ao hospedeiro e oportunista. Os efeitos da infecção, nomeadamente a diarreia, desidratação e desequilibrios electrolíticos, juntamente com acidose metabólica, enfraquecem os animais e podem originar elevados níveis de morbilidade e mortalidade moderada, especialmente quando associado a outros agentes.

Actualmente não é conhecido nenhum tratamento específico completamente eficaz no combate à criptosporidiose bovina. O tratamento baseia-se na terapia de suporte por meio de fluidoterapia oral e endovenosa. Medidas preventivas assentes em melhorias de higiene e administração correcta de colostro são consideradas as mais importantes.

O potencial zoonótico do parasita, especialmente em indivíduos imuno-deprimidos, levanta preocupações de saúde pública.

No presente trabalho, foram elaborados 2 estudos clínicos, tentando confirmar a existência, prevalência e sinais clínicos de criptosporidiose em regiões na Alemanha e em Portugal.

Na Universidade Justus-Liebig em Giessen, Alemanha, 35 vitelos foram examinados tendo em conta os seus sinais clínicos. Vinte e dois dos animais examinados demonstraram sinais de diarreia e, entre estes, 36% excretavam oocistos. Seguiu-se um exame mais detalhado dos animais infectados, incluindo o estudo dos sinais clínicos e dos valores laboratoriais. Os sinais clínicos e laboratoriais detectados confirmaram os esperados (característicos da criptosporidiose), incluindo diarreia fluida, desidratação, fraqueza e hipotermia; acidose metabólica, aumento dos níveis de lactato e hiponatremia. Todos os vitelos recuperaram após fluidoterapia, controlo da acidose e antibioterapia. Lactato de halofuginona foi administrado em alguns animais mas não demonstrou efeitos evidentes.

Em Portugal, 30 vitelos foram examinados numa exploração de engorda na área do Ribatejo, considerando os seus sinais clínicos e a presença de oocistos após análise pela técnica de Ziehl-Neelsen modificada. A prevalência foi elevada (43%). Os sinais clínicos variaram e a excreção de oocistos nem sempre estava acompanhada de diarreia (portadores assintomáticos). A evolução de quinze vitelos re-examinados variou, evidenciando por um lado a existência do carácter autolimitante (3 vitelos melhoraram) e por outro um possível aumento da mortalidade (2 vitelos morreram) associados à doença.

Palavras-chave: *Cryptosporidium parvum*, diarreia, vitelos, desidratação, fluidoterapia, colostro.

Abstract: Cryptosporidiosis in pre-weaned calves – Observations in Germany and Portugal

Cryptosporidiosis is one of the most important causes of bovine neonatal diarrhea, being considered an important threat to bovine production and a cause of economical losses.

Pre-weaned, neonatal calves are particularly prone to the infection and development of clinical signs due to the protozoan parasite *Cryptosporidium parvum*, as they are born almost completely deprived of natural immunity.

This parasite is a worldwide existing, ubiquitous, non host specific and opportunistic agent of diarrhea. Dehydration and electrolyte imbalances, coupled with metabolic acidosis, weaken the animals and may cause high morbidity rates and moderate mortality, especially when occurring in combination with other infectious agents.

No completely effective specific treatment for calf cryptosporidiosis is known to this date. Treatment is mainly based on supportive care by oral and intravenous fluid therapy. Prevention by hygiene measures, correct colostral administration and management seem to be most important.

Public health concerns arise due to its zoonotic potential, especially in immune compromised individuals.

In the present dissertation, two case studies were developed, trying to confirm the existence, prevalence and clinical signs of cryptosporidiosis in Germany and Portugal.

In the Clinics for Obstetrics, Gynecology and Andrology with veterinary ambulance from the Justus Liebig University Giessen, Germany, 35 calves were examined for their clinical signs. 22 of the examined calves showed diarrhea and of these, 36% were positively excreting oocysts. A more detailed examination of the infected calves was performed and clinical signs and laboratory values studied. Detected clinical and laboratory signs could be confirmed by the expected (characteristic signs of cryptosporidiosis), including watery to thin mushy diarrhea, dehydration, weakness and hypothermia; metabolic acidosis, increased levels of lactate and hypernatremia. All calves recovered after alkalinizing fluid therapy and antibiotherapy. Halofuginone lactate was administered to some animals but it was not clear if it caused a major improvement in the recovery.

In Portugal, 30 calves were examined in a fattening unit in the Ribatejo area concerning their clinical signs and presence of oocysts after modified Ziehl-Neelsen technique.

Prevalence was high with 43% of animals showing oocysts in the faeces. Clinical signs varied and excretion was not always accompanied by diarrhea (assympthomatic carriers). Fifteen of the examined calves were reexamined one month later. Results varied greatly, evidencing on one hand the self-limiting character of the disease (3 calves improved) and, on the other hand, the possible connection of the disease to an increased mortality (2 calves died).

Keywords: *Cryptosporidium parvum*, diarrhea, calves, dehydration, fluid therapy, colostrum.

Contents

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List of abbreviations:

18S r DNA : sequence of recombinant DNA

Acetyl-CoA: Acetyl Co enzyme A.

AIDS: Acquired immune deficiency syndome

AMDS: Arthromyodisplasia syndrome

BD: Base deficit

BE: Base excess

BID: *bis in die* "twice daily"

BW: Body weight

cAMP: cyclic adenosine monophosphate

Cl^-: Chloride ion

CMT: California mastitis test

CNS: Central Nervous System

CO_2: Carbon dioxide

CSF: Cerebrospinal fluid

ECF: Extracellular fluid

e. g.: *exempli gratia*. "example given"

H^+: hydrogen ion

H_2CO_3: Carbonic acid

H_2O: water

HACCP: Hazard analysis and critical control points

HCO_3^-: Bicarbonate ion

Hsp 70: 70 kilodalton heat shock protein

i. e.: *id est.* "that is"

Ig: Immune globulin

IM: Intramuscular

INF- γ: Interferon-gamma

IPM: Integrated pest control

IV: Intravenous

K^+: Potassium ion

Kg: kilogram

Na^+: Sodium ion

NaCl: Sodium chloride (salt)

NSAID: Non-steroidal anti-inflammatory drug

OH: hydroxide

PGE_2: Prostaglandin E_2

PGI_2: Prostaglandin I_2

RT-PCR: Reverse transcriptase polymerase chain reaction

SIBO: Small intestine bacterial overgrowth

SID: Strong ion difference

SID: "once daily"

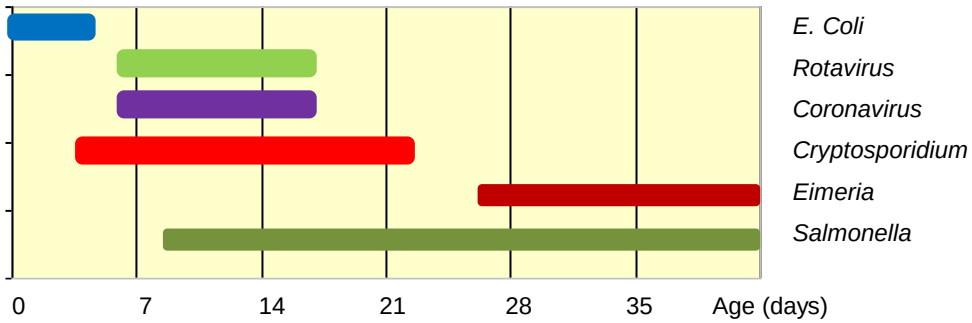
TNF-1: Tumour necrosis factor 1

$ZnCl_2$ – NaCl: Zinc chloride and sodium chloride solution

Introduction:

"Infectious diarrhea remains one of the biggest health challenges in both the beef and dairy industries." (page 13, Foster & Smith, 2009).
In dairy farms 50% of the calf mortality is assigned to diarrhea and in beef cattle it is thought to have an important impact in economic productivity (Foster & Smith, 2009).
Taking this fact into account, it is important to enlarge the knowledge of the causes, consequences, signs, therapy and prevention of this morbid condition.

There are innumerable causes that originate diarrhea in calves, some of them with worse consequences than others and hence some of them more urgent in matter of treatment and prevention.
Diarrhea can be infectious or non-infectious in its origin. Non-infectious diarrhea is normally associated to feeding mistakes (such as mixing or temperature errors). Infectious diarrhea is, on the other hand, mainly caused by four agents: enterotoxigenic *Escherichia Coli* (ETEC), *Rotavirus, Coronavirus* and *Cryptosporidium parvum* (Kaske & Kunz, 2003; Foster & Smith, 2009). Most of these agents affect calves in their first 4 weeks of life (Berchtold, 2009).



Graph. 1: Most frequently encountered infectious agents in neonatal calves displaying the most affected age-groups.
Addapted from: Kaske & Kunz (2003).

In this thesis, special attention will be given to a frequently encountered cause: Cryptosporidiosis.
"*C. parvum* is recognized as one of the most common infectious causes of intestinal disease in neonatal calves, lambs and kids…" (O'Handley & Olson 2006; de Graaf *et al.*1999b). As a very prevalent agent, it is thought to be present in almost every region worldwide (Fayer, Trout & Jenkins, 1998; Naciri, Lefay, Mancassola, Poirier & Chermette, 1999; Tzipori & Ward, 2002).

Cryptosporidium is a protozoan, unicellular, opportunistic and worldwide existing parasite that affects a wide range of hosts. It was found that neonatal and pre-weaned calves are especially susceptible to the infection.

When a calve is born, its immune system is still very weak, given the fact that most of the immunity in new born calves will be attained after birth, through the ingestion of the maternal colostrum (Kaske & Kunz, 2003). In addition to the weak immunity, at birth a calve has to cope with the changes of environment by adapting its respiratory and cardiovascular systhems, thermoregulation and other organic functions to the extrauterine environment and by dealing with the increased infective factors upon leaving the sterile intrauterine surroundings (Kaske & Kunz, 2003).

Given these risk factors, it becomes more understandable that calves are highly susceptible to infectious agents, including *Cryptosporidium* spp.

Special attention should be given to any alterations in their health status in their first days and weeks of life, detecting any signs that indicate a sick calf. The table no. 1 in the attachments shows the most important signs observed in a healthy calf.

The first description of *Cryptosporidium* was performed by Tyzzer, who identified it in a laboratory mouse in 1907. Only about 70 years later, the real significance of the parasite as a potential pathogenic agent became known, also when *C. parvum* was first characterized as a potential agent able to infect humans, in 1976 (Saini, Ransom & McNamara, 2000; Tzipori & Ward, 2002; O'Hara & Chen, 2011). In the 80's its importance as a primary cause of diarrhea and as a potential threat for immune compromised individuals (AIDS) became evident (Tzipori & Ward, 2002; O'Hara & Chen, 2011). And in 1993, finally, it was characterized as "the most serious, and difficult to control, cause of waterborne-related diarrhea" (page 1047, Tzipori & Ward, 2002; by McKanzie *et al.* 1994).

Infection by *Cryptosporidium* spp. in cattle was first completely reported in the beginning of the 70's (Saini *et al.* 2000; Tzipori & Ward, 2002 - described by Morin, Lariviere & Lallier (1976) and Pohlenz, Moon, Cheville & Bemrick (1978)). Its role as a primary pathogen was first questionable as it usually coexisted with other enteropathogens. Only in the 80's the role as a primary cause for neonatal diarrhea in calves was discovered by Tzipori *et al.* (de Graaf, Vanopdenbosch, Ortega-Mora, Abbassi & Peeters, 1999b; Naciri *et al*. 1999). Studies in the 1980's revealed that the infection was much more prevalent in neonatal calves than thought before. Reaching epidemic to endemic level in some cases, it was by then recognized as an important cause of neonatal calf diarrhea (de Graaf *et al.*1999b; Tzipori & Ward, 2002; Divers & Peek, 2008).

Nowadays, even with the high number of studies performed, this parasite continues to be rather enigmatic. Studies continue to be of difficult performance because the propagating of

the parasite *in vitro* continues to be impossible on a continuous matter and because of the inability to cryopreserve this microorganism (Tzipori & Ward, 2002).

The present essay describes the most important facts about this parasite, focusing on biology, clinical signs, diagnosis, treatment, prevention and public health issues.

Two clinical case studies were performed, in the Ribatejo area of Portugal and in the region of Hessen in Germany, in order to understand the reality of the infection and its clinical course.

The internship and the performed activities:

The official curricular internship that gave rise to this dissertation had a total duration of 4 months, beginning on the 1st of October 2010 and lasting until the 31st of January 2011. It was spent in two different locations, the first part being at the Clinic for Ruminants – Internal Medicine and Surgery (Klinik für Wiederkäuer – Innere Medizin und Chirurgie) (around 340 Hours) and the second at the Clinic for Obstetrics, Gynecology and Andrology with veterinary ambulance (Klinik für Geburtshilfe, Gynäkologie und Andrologie der Gross- und Kleintiere mit Tierärztlicher Ambulanz) (around 380 Hours, including nightshifts) at the Justus Liebig University Giessen, Germany. The total duration of the internship was, thus, of around 720 hours.

In addition, an extracurricular internship with the duration of around 260 hours was spent from the 3rd of March to the 9th of April 2011 at the Veterinary Clinic Karsten von Brehm in Niesgrau, Germany.

In the Clinic for Ruminants, under the supervision of Dr. Marlene Sickinger, several clinical and diagnostic methods were applied.

Fig. 1 and 2: Claw trimming (left) and CMT test and udder examination (right). (Original pictures).

Diagnostic methods included: general physical examinations and special examination of the respiratory tract, the gastro-intestinal tract, the circulatory system, the musculoskeletal system, the urinary tract and the nervous system: auscultation of the heart and lung, determination of the heart and respiratory rate, rumen activity auscultation, reticular pain probes, abomasal displacement test (steel band effect, lung percussion and ballottement), intestinal activity, fecal analysis (parasite, bacteria, virus), ruminal fluid quality testing (diagnosis of ruminal acidosis), rectal palpation with focus on the abdominal organs (rumen, intestines, kidneys, vessels and lymph nodes), diagnosis of udder disorders (CMT test, udder and milk evaluation), facial reflexes evaluation, urine testing (direct collection or via urinary catheter), ketosis diagnosis, blood analysis, cerebrospinal fluid collection, bone marrow collection, X-ray, amongst others.

Fig. 3, 4, 5 and 6: Blood sample collection (up left), Claw amputation and recovery (up right), Right abomasal displacement solution by the Dirksen method (bottom left), Positioning for left abomasal displacement solution by the Jannowitz method (bottom right) (Original pictures).

Therapeutic methods included: vaccinations, injections of all type (subcutaneous, intramuscular, intravascular, intraperitoneal, epidural, intradermic), ear vein and jugular vein

catheterization, infusions, whole blood transfusions, oral administrations, drenching (ruminal fluid transfer, ruminal stimulants), bandaging and fracture treatment, claw trimming, euthanasia.

I also was able to assist in surgical procedures such as the surgical resolution of left abomasal displacement (by the Jannowitz method), surgical resolution of right abomasal displacement (Dirksen method), ruminotomy and production of a ruminal fistula, ceacal tympany resolution, claw amputation, deep digital flexor tendon tenotomy, rectal prolaps solution in an Alpaca.

The second part of the internship in the clinic of Reproduction, Obstetrics and Andrology was done under the supervision of Prof. Dr. Axel Wehrend. Different to the previous clinic, where only ruminants were treated, in this clinic in addition to ruminants, horses, pigs, dogs, cats and rabbits where present.

Diagnostic methods included similar ones to the learned before, but major focus was given to the examination of the reproductive tract, including vaginal specula positioning with mucosal and cervix evaluation, semen collection and evaluation, rectal palpation and determination of reproductive status, vaginal palpation, fetal position evaluation, transrectal ultrasound in cows, abdominal ultrasound to determine vitality of the lamb in sheep, ultrasound of the

Fig. 7 and 8: Lamb after assisted birth (left), critical care in a calf (right) (Original pictures).

teats, uterine cytology and biopsy in cows and mares, mastitis evaluation, evaluation of calves with diarrhea, respiratory conditions, septicemia, articular fluid collection, amongst others.

Implemented treatment methods included all type of injections, infusion therapy, teat injectors, estrus synchronization, teat amputation, theloresectoscopy, birth assistance in cows, sheep, horses and pigs, castration of pigs and rams, cesarean sections (in cows, sheep, sows and dogs), fetotomy in cow and mare, resuscitation and critical care of calves, Bühner band in sheep, umbilical surgery in calves, bandaging of calves suffering from AMDS (Arthromyodisplasia syndrome), tenotomy to solve AMDS, approach to calve diarrhea and respiratory diseases (antibiotherapy, pain management, supportive treatment), acupuncture and homeopathy.

Fig. 9 and 10: Inhalation of saline solution in a calf suffering from respiratory disease (left), Arthromyodisplasia syndrome bandaging (right) (Original pictures).

Fig 11: Preterm piglet (Original picture).

Further performed activities included: feeding techniques of calves, farm visits and evaluation, euthanasia.

During the stay in the Clinic for obstetrics, ginecology and andrology, several calves suffering from diarrhea were identified, evaluated and tested for the presence of *Cryptosporidium* oocysts in their feces. The detailed case study and its results are outlined in greater detail elsewhere in this essay.

In the extracurricular internship under the supervision of Dr. Karsten v. Brehm, in contrast to the 2 previous internships, the activities consisted mainly of direct veterinary work at the farms.

The activities included basic diagnostic methods, injections, infusions, antibiotherapy, artificial inseminations, estrus synchronization, pregnancy diagnosis, rectal examination of the reproductive tract of cows, birth assistance in cows and sheep, resuscitation and critical care of calves, fetotomy in cows, left sided abomasal displacement solution (Laparoscopic abomasopexy technique by Christiansen), fecal analysis, diarrhea treatment, euthanasia, ketosis treatment, hypocalcemia and hypophosphatemia treatment, hypomagnesiemia treatment, ruminal bloat in calves, vaccination programs, milk sampling and mastitis treatment.

In Lisbon another clinical case study was performed with examination and fecal sample collection of 30 calves and posterior analysis of the samples in the parasitology laboratory of the Faculty of Veterinary Medicine – UTL Lisbon.

Literature review

The Parasite:

Parasites of the *Cryptosporidium* genus are well-known agents of ruminant's disease and have been deeply reviewed over the past years.

Cryptosporidium is classified as an ubiquitous, obligate, intracellular parasite, which affects all domestic animals and even humans (Naciri *et al*. 1999; Fayer, Morgan & Upton, 2000; Kaske & Kunz, 2003; Hamnes, Gierde & Robertson, 2006).

Studies conducted by Naciri, *et al*. (1999) showed that Cryptosporidium is one of the major etiological agents in neonatal calves from 4 to 10 days of age. Blume (2007) also evidenced *Cryptosporidium* as the most common cause of diarrhea in neonatal calves.

These parasites belong to the Kingdom Protozoa, phylum Apicomplexa, class Sporozoea, subclass Coccidia, Order Eucoccidiida, suborder Eimeriina and family Cryptosporidiidae (Rommel, Eckert, Kuntzer, Körting & Schnieder, 2000; Tzipori & Ward, 2002).

Comparing members of the cryptosporidiidae family with other coccidia, it becomes evident that they have many similarities in their life-cycle (presence of both sexual and asexual forms) (Divers & Peek, 2008). However, unlike other coccidian, *Cryptosporidium* oocysts are excreted in the feces in the already sporulated form and can cause auto-infection (de Graaf *et al.* 1999b; Tzipori & Ward, 2002; Hamnes *et al.* 2006; O'Handley & Olson, 2006). They differ in their host specificity, *C. parvum* being much less host specific, and in their size (*Cryptosporidium* spp. is much smaller in size and is much more difficult to detect by traditional fecal flotation methods) (Tzipori & Ward, 2002; Divers & Peek, 2008). Their high resistance as well as their characteristic location in the cell, are two additional features which differentiate *Cryptosporidium* from other coccidians (Tzipori & Ward, 2002; Hamnes *et al*. 2006).

In the last years, 13 valid species of *Cryptosporidium* were described (Olson, O'Handley, Ralston, McAllister & Thompson, 2004; Thomson, Palmer & O'Handley, 2007). Recently, further *Cryptosporidium* species have been identified, so nowadays the number of species rose to 23, and further non-identified species might exist (Muhid, Robertson, Ng & Ryan, 2011).

We can highlight 6 main species of cryptosporidia that affect animals. Those are: *C. parvum* and *C. andersoni* (mammalian), *C. maleagridis* and *C. baileyi* (avian), *C. serpentis* (reptiles) and *C. nasorum* (fish).The species from dogs (*C. canis*), cats (*C. felis*), pigs (*C. suis*) guinea pigs (*C. wrairii*) and marsupials are less well defined (Tzipori & Ward, 2002; Thompson *et al*. 2007).

Because most of the diagnosis were made by microscopy, to this date many unknown species may exist which were wrongly classified as *C. parvum* (because of the absence of morphological differences) (Thompson & Smith, 2011).

There are 3 main species of cryptosporidia that have an impact in the bovine species (Fayer, Santín, Trout & Greiner, 2006; Hamnes *et al.* 2006; O'Handley & Olson, 2006):

Cryptosporidium parvum is a pathogenic, non host specific, unicellular parasite that colonizes the small and large intestine of over 150 animal species, including all domestic animals (calves, lambs and young pigs especially) and also humans (Kaufmann, 1996). It is the most extensively studied because of its zoonotic potential as well as its clinical relevance as an important cause of neonatal diarrhea in ruminants, where it usually colonizes the ileum and the proximal large intestine (Laurent, McCole, Eckmann & Kagnoff, 1999; Naciri *et al.* 1999; Rommel *et al.* 2000; O'Handley & Olson, 2006).

Cryptosporidium andersoni (once called *muris*) can be found in the mammal's stomach (abomasum of ruminants). At this site, it causes dilation of the gastric glands, hypertrophy of the mucosa and atrophy of the epithelium. The protein digestion seems to be impaired (Olson *et al*. 2004). Even though it was declared to be non pathogenic, it can be held responsible for economic losses in both beef and dairy industry and is nowadays seen as a possible emerging pathogen. Its global prevalence is considered to be low (2 – 5%) (Santín *et al*. 2004) and the disease seems to be limited to cattle. Affecting primarily post-weaned beef and dairy cattle as well as mature animals, it is classified as a chronic infection (with a clinical course of months or even years) (Olson *et al*. 2004; Santin *et al*. 2004; Fayer *et al*. 2006; O'Handley & Olson, 2006). Pre-weaned calves can be already infected at an early age with this pathogen, but only show clinical signs when they are older (Kváč, Hromadová, Květoňová, Rost & Sak, 2011).

A new species of *Cryptosporidium* (*C. bovis*) was isolated in the USA (Divers & Peek, 2008). Encountered usually at calves aged from 2 to 7 months (Fayer *et al.* 2006, O'Handley & Olson, 2006), it is not certain yet if it is involved in neonatal diarrhea in calves (Divers & Peek, 2008).

A deer-like genotype (*C. ryanae*) was identified and can occur predominantly in weaned calves aged 2 to 11 months, without showing clinical signs (Fayer *et al.* 2006; Kváč *et al.* 2011).

Despite the multiplicity of *Cryptosporidium* species, one species remains the most important in mammals: *Cryptosporidium parvum* (Tzipori & Ward, 2002). Especially in pre-weaned calves, *C. parvum* is the most prevalent species and the only one with clinical relevance (Mendonça *et al*. 2007; Divers & Peek, 2008; Imre *et al.* 2011; Kváč *et al*. 2011; Muhid *et al.* 2011). To avoid the creation of a too extensive essay, major focus will be given to this species of *Cryptosporidium* and future reference to the disease will be concerning *C. parvum* infection.

Morphology and life-cycle:

The oocysts of *Cryptosporidium parvum* are circular shaped, with a size of approximately 5,0 x 4,5 µm. A double layered lipoprotein membrane, forming the oocyst membrane, encloses 4 sporozoites and a crystalline residual body (de Graaf *et al.* 1999b; Rommel *et al.* 2000).

C. parvum develops in the small intestine, especially in the caudal part of the jejunum and in the ileum and sometimes also in the large intestine (de Graaf *et al*. 1999b; Rommel *et al*. 2000). All developmental stages are localized in the apical pole of the epithelial cells, but even though it multiplies in the cell, it is not included in the cell cytoplasm (O'Handley & Olson, 2006). Hence, they are localized intracellularly but extracytoplasmatically (Heine, Pohlenz, Moon & Woode, 1984; Kaske & Kunz, 2003).

The whole development of the parasite is located in one single host, classifying the life cycle as monoxenous (de Graaf *et al.* 1999b; O'Hara & Chen, 2011).

The infection is usually carried out through the fecal-oral route, by ingestion of oocysts in the feces (Rommel *et al.* 2000; Tzipori & Ward, 2002; Hamnes *et al.* 2006; Coklin *et al.* 2009). In addition, contaminated ground water and feedstuffs can be an important source of infection (Hamnes *et al.* 2006; Divers & Peek, 2008; Coklin *et al*. 2009); as are rubber and plastic fomites which, when insufficiently cleaned, can be a way of transport for the infective oocysts (Bopp, 2003). In general, it is believed that farm effluents are probably the most important sources of environmental contamination with oocysts (Tzipori & Ward, 2002).

Fonseca (2000) confirmed the importance of transmission of cryptosporidiosis by the drinking water, as 55% of the analyzed water samples in a study in Portugal contained *Cryptosporidium* oocysts.

Assymptomatic adult cattle are rather frequent and can easily contribute to the spreading of the infection to young calves by excreting the infectious oocysts (de Graaf *et al.* 1999b; Naciri *et al*. 1999; Tzipori & Ward, 2002). This is thought to be the most important source of *C. parvum* infections in dairy calves that suckle the mother after birth (Tzipori & Ward, 2002). The contact to wildlife species carrying the same parasite to which also bovine species are susceptible constitutes another possible way of infection (Bopp, 2003; Olson *et al.* 2004).

After ingestion of the sporulated oocysts, the changes in temperature and pH (gastric acid), as well as the concentration of carbon dioxide, pancreatic enzymes and bile salts act on the oocysts and lead to their excystation (Heine *et al*. 1984; Laurent *et al.* 1999; de Graaf *et al.* 1999b; Tzipori & Ward, 2002; O'Handley & Olson, 2006; Foster & Smith, 2009). Parasite endogenous factors probably contribute to the successful excystation mechanism. These include: serine and cystine proteases associated to the sporozoites, secretory phospholipase A_2, molecules associated with protein synthesis (proteins related to ribossomes or heat-shock proteins), arginine aminopeptidase (O'Hara & Chen, 2011).

This way the 4 motile and infective sporozoites of *C. parvum* are freed into the gut lumen and start colonizing areas between microvilli in the small intestine, mainly in the ileum (sporadically also in the colon) (Tzipori & Ward, 2002; Gookin, Nordone & Argenzio, 2002; O'Handley & Olson, 2006; Foster & Smith, 2009). The fusion of the parasite's pellicula with the microvilli membrane and invagination of the luminal membrane follows (forming the parasitophorous vacuole which has an extracytoplasmic but intracellular location), with the simultaneous formation of feeder organelle between the parasite and the cellular cytoplasm (Laurent *et al*. 1999; Gookin *et al.* 2002; Tzipori & Ward, 2002; Foster & Chen, 2009; O'Hara & Chen, 2011). *C. parvum* is thought to be completely dependent of the nutrient support by the host through the feeder organelle, as the parasitic biochemical mechanisms are rather underdeveloped (O'Hara & Chen, 2011).

The trophozoite is formed and undergoes asexual reproduction to give rise to a type 1 schizont (or meront) (5 x 5,6 µm), enclosing 8 banana-shaped merozoites (by a process called endopolygeny) (Rommel *et al*. 2000; Tzipori & Ward, 2002; Gookin *et al*. 2002; O'Handley & Olson, 2006; Foster & Smith, 2009; O'Hara & Chen, 2011).

After rupturing, the merozoites are freed in the gut lumen and form either new type 1 schizonts on other sites or type 2 schizonts which enclose only 4 merozoites. From the latter, micro- and macrogametocytes are formed in which, respectively, 16 microgamettes or a 4,6 µm large macrogamette is formed (Rommel *et al.* 2000; Tzipori & Ward, 2002; Gookin *et al.* 2002; Foster & Smith, 2009; O'Hara & Chen, 2011).

A diploid zygote is formed when the non-flagelated microgamete fertilizes the macrogametocyte (Tzipori & Ward, 2002; Gookin *et al.* 2002; O'Handley & Olson, 2006; O'Hara & Chen, 2011). The sporogony process (a mechanism similar to meiosis) originates the sporulated oocyst (enclosing 4 haploid sporozoites). This structure is either excreted to the environment (thick walled oocysts) after a prepatent period of 3 to 6 days or originates an autoinfection if thin walled oocysts are formed. The thin walled oocysts, instead of being excreted to the environment, are freed still inside the host, restarting the whole endogenous cycle (Rommel *et al*. 2000; Gookin *et al.* 2002; Foster & Smith, 2009; O'Hara & Chen, 2011). The excreted oocysts are immediately infective to other animals when they are ingested and remain so for a long period of time in the environment (Gookin *et al.* 2002; Foster & Smith, 2009; O'Hara & Chen, 2011).

This autoinfection by thin walled oocysts is thought to be the cause of extended clinical conditions which, more likely, may lead to cachexia and death of the newborns (Tzipori & Ward, 2002; Divers & Peek, 2008).

The shedding begins often at 3 days of age and peaks at 14 days. The animal usually shows clinical signs at 3 to 5 days and during 4 to 17 days, being often able to excrete up to 10^7 oocysts per gram of feces (Fayer *et al.* 1998; Olson *et al.* 2004; Hamnes *et al.* 2006; Radostits, Gay, Hinchcliff & Constable, 2007).

Usually the infection remains restricted to the gastrointestinal tract, however there can be cases of extra-intestinal phases in other organs (Tzipori & Ward, 2002).

Epidemiology:

Cryptosporidiosis is a frequent disease in neonatal ruminants occurring world-wide, especially in dairy calves.

Longitudinal studies indicated that the incidence in dairy herds could easily reach 100%, whereas in beef herds the incidence was much lower (3 – 25%) (Jäger *et al.* 2005; Gow & Waldner, 2006; O'Handley & Olson, 2006).

The prevalence in dairy calves was determined in studies conducted by de la Fuente *et al.* (1999) (Spain), Almeida, Oliveira & Teixeira (2008) (Brasil), Kváč *et al.* (2011) (Czech Republik) and Imre *et al.* (2011) (Romania). Prevalences in calves ranged from 25 to 61%. In a study by Coklin *et al.* (2009) (Canada), however, prevalence in dairy calves reached only 6%.

A study of the prevalence of infection by *Cryptosporidium* spp. in beef calves was conducted by Ralston, Mc Allister & Olson (2003) in Canada and did not exceed 5%. Gow and Waldner (2006) detected a prevalence of 3,1% amongst beef-calves in Canada, and Jäger et al. (2005) a prevalence of 20 – 25% in beef-calves in Germany.

In the study in France, conducted by Naciri *et al.* (1999), a higher prevalence in suckling compared to dairy calves was detected, which somehow contradicts the previously established facts.

Dairy calves may have a higher incidence of cryptosporidiosis because the beef calves are born in a single period in spring while dairy calves are born all year round. So, considering their short calving season, the transmission is much lower in beef than in dairy calves. Dairy calves are housed in pens that are kept close together with other animals, whereas beef calves are released to a large open area (Olson *et al.* 2004).

Even though it was thought that the lower prevalence in beef calves is related to the lower stock density when comparing to dairy calves, recent studies showed that even when keeping beef calves in a similarly confined environment as dairy calves, the incidence in beef calves never was higher than 25%, which strongly suggests other factors that influence the different susceptibility in beef and dairy calves (Kaufmann *et al*. 1996; Jäger *et al*. 2005).

The necessary dose for a successful infection is very variable and depends on the individual resistance of the host. Nevertheless, susceptible or immune-compromised animals need a very low dose (as low as 10 oocysts sometimes) to get infected (Divers & Peek, 2008; O'Hara & Chen, 2011). As an infected calve sheds several million oocysts in the diarrheic feces (up to 10^7), the concentration and risk of infection in an affected farm is very high

(infective pressure) and the problem can easily turn into a herd problem (de Graaf *et al.* 1999b; Hamnes *et al.* 2006; Divers & Peek, 2008).

In addition to the low infectious dose, another feature that aids the transmission is the extremely high resistance of the oocysts in the environment, mainly due to their highly protective carbohydrate wall (Rommel *et al.* 2000; Bopp, 2003). At 4ºC with a sufficient degree of humidity, they can survive for 6 months, only being destroyed immediately at temperatures under – 18º C or over 65º C (Naciri *et al.* 1999; Rommel *et al.* 2000). Standard levels of chlorination are also insufficient to destroy the oocysts, allowing an easy infection through the waterborne way in animals and humans (Betancourt & Rose, 2004; Olson *et al.* 2004). The presence of chloride can even increase the infectivity of the oocysts, as it can hasten the excystation (Fonseca, personal communication on the 26th January 2012).

A relation between the incidence of cryptosporidiosis and the season of the year has been suggested, but no significant correspondence has been identified. In winter, when the confinement of the calves is higher in some countries, it might occur more often (Hamnes *et al.* 2006, Radostits *et al.* 2007).

Clinical signs:

The clinical signs of a *C. parvum* infection are nonspecific and can not be easily differentiated from bacterial or viral infections that cause diarrhea. They are similar in calves, lambs and kids and include diarrhea, dehydration, abdominal pain, reduced appetite and depression (Naciri et *al.* 1999; de Graaf *et al.* 1999b; Olson *et al*. 2004; O'Handley & Olson, 2006; Divers & Peek, 2008; Kváč *et al.* 2011). Anorexia, combined with the diarrhea, usually gives rise to weight loss and retarded growth in affected calves (de Graaf *et al.* 1999b).

Following the study from Vos, Constable & Kuhlenschmidt (2005) (USA), abomasal emptying in cases of a *Cryptosporidium parvum* infection was increased at the beginning of infection, resuming to a normal rate 1 week after the start of the diarrhea.

The infection is characterized by profuse and persistent watery, yellow-greenish colored diarrhea with putrid smell, containing mucus (Kaufmann *et al*. 1996; de Graaf *et al.* 1999b; Rommel *et al.* 2000; Olson *et al*. 2004; O'Handley & Olson, 2006; Radostits *et al.* 2007; Thompson *et al.* 2008). These clinical signs can appear in animals as young as 3 days of age, rarely affecting calves of 30 days and older (Santín *et al.* 2004; Fayer *et al*. 2006). Mostly calves with 5 to 14 days of age are affected and the infection lasts for approximately 4 to 18 days (Rommel *et al.* 2000; Kaske & Kunz, 2003; Olson *et al.* 2004; O'Handley & Olson, 2006; Thompson *et al.* 2008). Even though it occurs rarely, the signs are less severe if the infection occurs in older (over 4 weeks of age) calves (Rommel *et al.* 2000, Hamnes *et al.* 2006).

Several studies carried out by Naciri *et al.* (1999) in France, de la Fuente *et al.* (1999) in Spain, Santín *et al.* (2004) in the USA, Mendonça *et al.* (2007) in Portugal, Muhid *et al.* (2011) in Malaysia, Imre *et al.* (2011) in Romania and Tiranti *et al.* (2011) in Argentina proved the higher incidence of cryptosporidiosis in preweaned calves in comparison to post-weaned ones. Being the most affected group, animals aged between 8 and 14 days.

Generally, when comparing to other agents (*E. Coli, Rotavirus* and *Coronavirus*), findings suggest that calves affected by *Cryptosporidium* spp. get sick later and during a longer time, but die less often. The degree of dehydration seems, though, higher in *Cryptosporidium* spp. infections (Blume, 2007).

Even though the parasite infects preferably the distal part of the small intestine, it can spread throughout the gut. In this way, the diarrhea is most watery and intense when the parasite colonizes the proximal parts of the small intestine, if the infection affects mainly the distal parts of the intestine, the infection might even be assymptomatic (Tzipori & Ward, 2002).

Some data suggests that older cattle can get immunized to future infections with this parasite because of previous exposure (Harp, Woodmansee & Moon, 1990; Fayer *et al.* 1998; Coklin *et al.* 2009). Studies conducted by Harp *et al.* (1990) showed that rechalenging calves that previously suffered from cryptosporidiosis, after 3 months, evidenced neither clinical signs related to cryptosporidiosis nor oocyst shedding.

Together with the signs of diarrhea, oocyst shedding takes place. The period of oocyst shedding, however, is determined by host factors (age and immune status). After the shedding begins, the number of excreted oocysts increases, reaching a maximum 5 to 6 days after the infection and decreasing suddenly 10 to 15 days after the day of infection (de Graaf *et al.* 1999b; Hamnes *et al.* 2006; Radostits *et al.* 2007). The excretion of oocysts can persist for several months even after disappearance of the clinical signs (de Graaf *et al.* 1999b; Naciri *et al.* 1999; Tzipori & Ward, 2002).

In a study conducted by Fayer *et al*. (1998) the prepatent period in infected calves ranged from 3 to 6 days and at the peak of infection (6-8 days post infection), calves were able to excrete up to 10^7 oocysts per gram of feces. Most of the calves excreted oocysts during 69 days.

Blume (2007) detected oocyst excretion already on the 3rd day of age in some animals and a study conducted by Vos, Constable & Kuhlenschmidt (2005) evidenced the oocyst shedding cyclic pattern (low in the beginning, increasing later on and decreasing again).

Morbidity of affected calves with less than 3 weeks of age can reach values greater than 50%, however mortality is usually low if supportive fluid therapy is sufficient and no mixed infections exist (Divers & Peek, 2008); as the main cause of death remains the high loss of fluids due to diarrhea and not the direct action of the infective agent (Kaske & Kunz, 2003).

In rare cases, severe cryptosporidiosis can lead to extreme dehydration, metabolic acidosis and death due to cardiovascular collapse (O'Handley & Olson, 2006; Radostits *et al.* 2007; Thompson *et al*. 2008).

Frequently the infection with *Cryptosporidium parvum* occurs together with other infective agents (mixed infection) (Naciri *et al.* 1999; de la Fuente *et al.* 1999; Kaske & Kunz, 2003; Radostits *et al.* 2007). In fact, in a study by Blume (2007), half of the collected fecal samples contained a mix of several diarrhea causing agents.

The reason for the mixed infections is that this parasite is mostly opportunistic and infects especially immune supressed organisms (Kaske & Kunz, 2003). In addition, lesions of the intestinal mucosa predispose the calf to the infection by other agents such as *E. Coli*, *Salmonella* sp. and viruses (especially *Rotavirus* and *Coronavirus*) (Divers & Peek, 2008).

The fecal samples are likely to contain several agents in addition to the Cryptosporidia. However, as *C. parvum* oocysts are shed throughout and even after the presence of clinical signs and *Rota* or *Coronavirus* are shed only in the beginning, *C. parvum* is more often found. Hence, the early collection and analysis of fecal samples is crucial for the correct determination of the etiology of the calves' diarrhea (Divers & Peek, 2008). *i.e. C. parvum* is a pathogenic entity by itself, whose symptoms can be worsened by the presence of other diarrhea causing agents (*E. coli, Corona virus, Rota virus*…) (Naciri *et al.* 1999; Rommel *et al.* 2000).

In case of a monoinfection with *C. parvum*, if the calf is not too weak and the density of infective forms in the environment is not too high, the infection is usually self-limiting and mild (Kaufmann *et al.* 1996; Divers & Peek, 2008). This is especially seen if the calves continue nursing (Divers & Peek, 2008).

In mixed infections, the clinical signs and prognosis tend to be worse (acidosis, electrolyte imbalances, dehydration and possible dysentery) and treatment is more complicated. Thus, there is usually a higher mortality rate (Divers & Peek, 2008).

As to the impact on production, to this date there are no data that support any long-term effects on production due to the infection with *C. parvum* in calves. No differences regarding weight gain have been noted between treated and untreated animal groups. Economic losses are, thus, only related to the cost of treatment and death of infected animals, which nevertheless can be substantial (Divers & Peek, 2008).

Pathogenesis / Pathophysiology:

The exact mechanism of pathogenesis of cryptosporidiosis is not well known to this date. Even though some structural evidences are understood, much research is still needed to know the molecular processes that are involved (Tzipori & Ward, 2002).

As mentioned before, *Cryptosporidium parvum* parasites affect essentially the distal small intestine (ileum and jejunum), but can be also found in the proximal small intestine and portions of the large intestine, colonizing especially the most apical part of the cells. Usually deeper mucosal layers are not invaded, hence the parasite is often classified as a "minimally invasive" mucosal pathogen (page 141, Laurent *et al.* 1999).

The pathologic changes in the intestine of an affected individual are caused mainly by the rupture of the parasitophorous vacuole with the consequent release and spreading of the merozoites to other enterocytes (Divers & Peek, 2008). These changes include villous atrophy, villous fusion, crypt hyperplasia, infiltration with inflammatory cells and destruction of microvilli (Koudela & Jiři, 1997; McCole, Eckmann, Laurent & Kagnoff, 2000; Gookin *et al.* 2002; O'Handley & Olson, 2006).

The severe villous atrophy is thought to be the result of the loss of the villous enterocytes followed by retraction of villi to conserve an intact epithelial gut barrier. In order to replace lost epithelial cells, the crypts undergo hyperplasia (due to acceleration of cell division), but disruption of the epithelial barrier can be observed in severe infections (Tzipori & Ward, 2002; Gookin *et al.* 2002; Foster & Smith, 2009).

Due to its fixation on the microvilli border, the parasite disturbs the normal function of the small intestine mucosa, not only by decreasing the absorbing area of the intestine (loss of intestinal mucosa), but also by disturbing the enzyme activity (loss of membrane bound enzymes), impairing the breakdown of peptides and dissacharides and the transmembranary nutrient and electrolyte transport (Rommel *et al.* 2000; Tzipori & Ward, 2002; Gookin *et al.* 2002; O'Handley & Olson, 2006).

Attachment and formation of the parasitophorous vacuole:

After excystation, the parasite attaches to the host cells and gives rise to the formation of the parasitophorous vacuole, by the action of several proteins lodged in the surface or apical complex (micronemes, rhoptries and dense granules) of the *Cryptosporidium* sporozoites. (Gookin *et al.* 2002; O'Hara & Chen, 2011).

The attachment, invasion and formation of the parasitophorous vacuole are complicated and intricate mechanisms. New discoveries identified several surface and apical complex proteins that are thought to be, in part, responsible for those mechanisms (CSL, GP900, p23/27, TRAP C1, GP15, CP 15, CP60/15, cp47, gp40/45 and gp15/Cp17) (Tzipori & Ward, 2002).

After a successful attachment, internalization occurs. The latter starts with the fusion of the parasite and the host cell membrane. When the rhoptry of the parasite contacts with the host cell attachment site and the micronemes and dense granules migrate to the merging area, a structure with a channel-like appearance forms and the cytoplasm of the parasite attaines a vacuolated form. As the vacuolization procedes, the parasite gets encapsulated by the host cell (O'Hara & Chen, 2011).

The sporozoites are internalized and the parasitophorous vacuole is formed (a double membrane formed by host cell and re-structured by the parasite, located intracellularly but extracytoplasmatically). At the host-parasite interface, an electron-dense structure with a polymerized actin network is formed (O'Hara & Chen, 2011).

Host cell actin branches are reorganized by several signaling axes and the epithelial membrane suffers a rearrangement which gives rise to the formation of transporter channels (for water and Na^+/Glucose movement, respectively). This way, *C. parvum* is thought to alter the cytoskeletal structure of the host cell upon the internalization process (O'Hara & Chen, 2011).

Diarrhea due to cryptosporidiosis is both malabsorptive and secretory (Laurent *et al.* 2009). Several mechanisms lye behind these processes and give rise to the clinical signs of cryptosporidiosis.

Malabsorptive diarrhea due to cell loss:

The cell loss could be an effect of the pathogen itself or a response from the host to limit and solve the infection (Foster & Smith, 2009). Hence, there are several possible mechanisms that lead to the extensive epithelial cell loss in *C. parvum* infections.

The first suggested mechanism consists of a direct cytotoxic effect of *C. parvum* on the epithelium of the intestine, by leakage of enzymes (lactate dehydrogenase). Nowadays this theory is not supported by current literature and is considered controversial (Laurent *et al.* 1999; McCole *et al.* 2000; Gookin *et al.* 2002; Foster & Smith, 2009).

The second, and more accepted, mechanism that explains cell loss is apoptosis (or programmed cell death). This theory is supported by the finding of apoptotic cells in *in vitro* and *in vivo* essays (Ojcius, Perfettini, Bonnin & Laurent, 1999; Chen *et al.* 2001; Foster & Smith, 2009). As shown recently, apoptosis is activated or inhibited in different stages of development of the parasite, occurring inhibition in early stages of the infection (trophozoite stage – when the parasite depends on the host cell for growth and development) and activation and increase of apoptosis in the later stages of infection (McCole *et al*. 2000; Foster & Smith, 2009; O'Hara & Chen, 2011).

Studies conducted by Mele, Morales, Tosini & Pozio (2004) showed that apoptosis is triggered by the invasion of the sporozoites in the first hours after infection (possibly by a caspase dependent mechanism), followed by an inhibition of apoptosis in the first 24 hours (probably by expression of antiapoptotic proteins). Then, 48 hours after the infection, apoptosis is again activated (to allow the exit of the merozoites from the cell).

Chen *et al.* (2001) suggested that *Cryptosporidium* actively inhibits the apoptosis of infected cells by secreting NF-ΚB, in order to protect the destruction of the infected cells. Meanwhile it also triggers the freeing of IL-8 which enhances the inflammatory reaction.

Ojcius *et al*. (1999) saw in his studies the role of *Cryptosporidium* in causing caspase-dependent apoptosis of infected cells. The suggested mechanism could be interpreted as a host action to limit *C. parvum* infection or an action from the parasite that helps it exiting the host cells.

It has to be discovered still if the referred regulation of apoptosis is beneficial for the host (limit spreading of the parasite, diminish extend of cell loss and/or increase clearance of the parasite from the host) or the parasite (preserve its intracellular location) (McCole *et al*. 2000; Chen *et al.* 2001; Gookin *et al*. 2002; Mele *et al.* 2004; Foster & Smith, 2009; O'Hara & Chen, 2011).

Secretory diarrhea due to prostaglandins:

A prostaglandin-mediated mechanism, responsible for the secretions of anions (mainly Cl^- and HCO_3^-) and the inhibition of the absorption of neutral NaCl, has been suggested in addition to the malabsoption mechanism described before (Laurent *et al.* 1999; Gookin *et al.* 2002; Foster & Smith, 2009). Two types of prostaglandins are involved: PGE_2 and PGI_2. These are originated most likely from macrophages that, infiltrating the lamina propria, activate prostaglandin secretion by mesenchymal cells. Nitric oxide may also play a role in stimulating prostaglandin-mediated secretion and is considered important in the defense against cryptosporidiosis. But the exact mechanism is not yet known (Laurent *et al.* 1999; Gookin *et al.* 2002; Foster & Smith, 2009).

PGE_2 and PGI_2 act on different sites, whereas PGE_2 exerts its function directly on the enterocytes, PGI_2 acts indirectly by influencing the enteric nervous system. 75% of the secretion in C. parvum infection is attributed to the effect of PGI_2. It stimulates the nicotinic ganglia as well as the VIP-ergic and cholinergic motor neurons responsible for the innervation of the intestinal mucosa (Laurent *et al.* 1999; Foster & Smith, 2009).

In the end, prostaglandin secretion stimulates calcium and cAMP freeing which decreases sodium absorption and increases anion secretion (Gookin *et al.* 2002).

INF-γ and TNF-1 contribute to the above mentioned mechanism, by increasing the overall permeability of the epithelium (Laurent *et al*. 1999).

The secretory mechanism is hence based on host immunity processes triggered by the infection.

In summary, the clinical signs associated with the disease are thought to be caused by a mixed mechanism including maldigestion, malabsorption and osmotic effects (malabsorptive and secretory diarrhea) (Divers & Peek, 2008).

The exact mechanisms of the pathogenesis are not yet fully understood and it seems that the underlying factors are originated both by the parasite and the host (O'Handley & Olson, 2006).

Diagnosis of cryptosporidiosis:

In cases of neonatal diarrhea in calves, cryptosporidiosis should always be included in the list of differential diagnosis (O'Handley & Olson, 2006).

To determine the presence of cryptosporidium, two main diagnostic methods are used: by analysis of the feces or by postmortem examination of the intestinal mucosa (Rommel *et al.* 2000). In both cases the diagnosis is done by microscopic observation of the oocysts in the samples (Divers & Peek, 2008).

In the clinically affected animals, the number of excreted oocysts is very high, which makes their detection in the feces easy (Tzipori & Ward, 2002).

Concentration of oocysts before observation under the microscope is a common method, the preferred method being the use of saturated sugar. Another method is the flotation in a $ZnCl_2$ – NaCl solution or other salts (Rommel *et al.* 2000; O'Handley & Olson, 2006; Radostits *et al*. 2007; Thompson *et al.* 2008).

Normal light microscopy makes the detection of the oocysts difficult as they can not be easily differentiated from the structures in the background. Phase contrast microscopy might be an alternative and allows differentiation of the oocysts (Radostits *et al.* 2007).

After concentrating the sample, staining methods can be applied to help the differentiation, those include the Ziehl-Neelsen technique (acid-fast stain, as cryptosporidium is an acid-fast organism), safranin-methylene blue stain, Kinyoun stain and DMSO-carbol fuchsin stain. All of these methods stain the oocysts red with a refractile area surrounding them and counter stains the background to facilitate the identification. These stains are quite helpful, but they are very time-consuming (Fayer *et al*. 2000; Rommel *et al.* 2000; Tzipori & Ward, 2002). The oocysts are seen as circular structures, 3 to 5 µm in size, often with 2-4 sporozoite nuclei in their interior, and can be easily differentiated from other microorganisms and fecal compounds (Rommel *et al*. 2000; Tzipori & Ward, 2002; O'Handley & Olson, 2006).

Negative staining with nigrosin, light green merbromide and malachite green, which stain all structures in the background except for the oocysts can be used but are not as trustworthy (Fayer *et al.* 2000).

As the oocysts often float, they will be lodged just under the coverslip of the microscope slide, in a high focal plane which, when focusing on them, will let other parasites (such as *Eimeria*) appear blurry (O'Handley & Olson, 2006).

Postmortem analysis of fresh necropsy tissue samples after fixation with methanol and staining of dab preparations with Giemsa (10% solution, 30 minutes staining) or of histological sections with Haematoxillin-Eosin, are two aditional diagnostic methods (Rommel *et al.* 2000).

At dissection, the intestine wall is thickened and hyperemic showing lesions of enteritis.

Histopathologic changes include atrophy and fusion of intestinal villi, especially in the distal jejunum and ileum (Laurent *et al.* 1999; Rommel *et al*. 2000; Radostits *et al.* 2007). Villi might be shorter, wider, blunt and might even appear fused. Crypts are hyperplastic and an inflammatory cell infiltrate of lymphoid cells, macrophages and neutrophils in the lamina propria is present (Laurent *et al.* 1999, Radostits *et al.* 2007). In the epithelial membrane, many parasitic forms are embedded (Radostits *et al.* 2007).

Immunological methods used in the identification of this parasite include: polyclonal fluorescent antibody tests, latex agglutination reactions, IF with monoclonal antibodies, ELISA (enzyme linked), reverse passive haemagglutination (RPH), immunocromatography, and several others (Fayer *et al.* 2000). These immunological methods may be quite accurate, but they are not routinely used as they are linked to rather high costs and because they can only be performed in specialized laboratories (Tzipori & Ward, 2002; O'Handley & Olson, 2006).

Coproantigen-based immunoessay kits (ELISA) can be used for a more sensitive and specific diagnosis of cryptosporidiosis. Their high price and low availability are factors limiting their application in routine cryptosporidiosis diagnosis, making them only useful in human cryptosporidiosis cases (Garcia & Shimizu, 1997; Thompson *et al.* 2008).

PCR and other genetic techniques (like the analysis of 18S rDNA, hsp70 and Acetyl- CoA synthethase) can be used to differentiate species of *Cryptosporidium* (which can not be attained by staining and microscopic observation) and bring great value in an epidemiological matter (determining of the source of a potentially zoonotic case) (Tzipori & Ward, 2002; Divers & Peek, 2008). PCR is a highly sensitive, accurate and quick method, however the number of false positives is rather high and it can be influenced by environmental factors (contaminants) (Fayer *et al.* 2000).

Viability and infectivity studies are possible to be carried out. Some examples of types of coloration include the vital dyes: propidium iodide and 4,6, diamino- 2´- phenylindole (DAPI). Viability can be also tested by fluorescent in situ hybridization (FISH) and cell culture followed by RT-PCR (Fayer *et al.* 2000).

As it was already mentioned, the infections are usually mixed, therefore, if the presence of a *C. parvum* infection is confirmed, diagnostic tests to determine bacterial or viral infections should be carried out (Divers & Peek, 2008).

In vitro studies of the parasite's life cycle show many restrictions in cell cultures. It usually has to involve several passages in hosts (mainly calves, due to their high susceptibility) (Fayer *et al.* 1998; Tzipori & Ward, 2002; O'Hara & Chen, 2011). Culturing was attempted in epithelial cell cultures (like HCT-8, a human ileocecal adenocarcinoma cell line), intestinal xenografts and by infection of animal models (e. g. mice) (Laurent *et al.* 1999).

In short, immunofluorescence, ELISA and PCR have a higher specificity and sensitivity for diagnosis, but are seldomly used. Routine diagnosis is mainly done by microscopical observation (Divers & Peek, 2008).

According to Fonseca (2000), the most efficient diagnostic method for cryptosporidiosis in calves was the enzyme immunoassay ELISA technique, which should be confirmed by a fecal smear stained by the Ziehl-Neelsen technique.

Therapy:

The location of *Cryptosporidium parvum* inside the extracytoplasmatic parasitophorous vacuole makes drug delivery difficult. Drugs that are administered, usually either pass directly through the lumen without penetrating the cell or they accumulate in the cells cytoplasm (Tzipori & Ward, 2002; Foster & Smith, 2009). The treatment is only thought to be efficient when the drug penetrates the cell's cytoplasm and parasitophorous vacuole or when the complete destruction of the affected cell is accomplished (Tzipori & Ward, 2002).

There is only one agent that is therapeutically effective and legally accepted in Europe: Halofuginone lactate (Kaske & Kunz, 2003). This compound, belonging to the group of the quinazolines, has still an unknown mode of action.

It is licensed for prevention of cryptosporidiosis in calves in the first 7 days of age (Koch, 2004; Foster & Smith, 2009). The commercial product known by the name of Halocur ®, contains 0.5 mg / mL halofuginone and should be given during 7 days, every 24 hours after milk feeding (60 – 120 µg/kg/day) (Kaufmann *et al.* 1996; Kaske & Kunz, 2003; Radostits *et al.* 2007) or mixed in at least 500 ml milk or milk replacer (Rommel *et al.* 2000). It reduces the severity and incidence of the initial clinical symptoms (diarrhea) and reduces and delays the excretion of oocysts (not preventing it completely) (Villacorta, Peeters, Vanopdenbosch, Ares-Mazás & Theys 1991; Kaske & Kunz, 2003; Olson *et al.* 2004; Jarvie *et al.* 2005; O'Handley & Olson, 2006). It should be used prophylactically when there is a problem in the stock, in the first 24 to 48 hours of life; excepcionally, animals that are already sick, can be treated at the latest 24 hours after the start of the diarrhea (Kaske & Kunz, 2003).

In general, however, oocyst excretion was reduced during the administration of halofuginone but resumed after its interruption, which suggests that it is rather cryptosporidiostatic (not killing the parasite) (Naciri, Mancassola, Yvoré & Peeters, 1992; Divers & Peek, 2008).

It is important to consider that halofuginone's therapeutical ratio is very low, which means that the dosage to reach the therapeutical effect is very close to the dosage that causes toxic effects. Thus, it is of major importance that the dosage is adjusted to the weight of the animal and that overdoses are avoided (Rommel *et al.* 2000). As it was proved by Villacorta *et al.* (1991), dosages in the range of 500 µg/kg/day can cause toxic side effects, such as apathy and bloody diarrhea (Villacorta *et al*. 1991; Kaske & Kunz, 2003).

As the official information regarding Halocur® indicates, the product neither should be used on an empty stomach nor in cases of diarrhea that lasted more than 24 hours. Halocur® seems to be potentially nephrotoxic in dehydrated and weak animals.

Skin allergies in farm workers are frequent. As a result, gloves should be used during its administration. For the environment, this product is equally harmful, because it can be dangerous for the aquatic flora and fauna and so should never enter any watercourses (MSD Animal Health, 2009).

Due to the high risk of halofuginone administration and due to the fact that it can only be used profilactically or in the first hours of the patent period, to this date there is no known specific and completely effective treatment of cryptosporidiosis in cattle.

Other drugs such as paromomycin, nitazoxanide and azithromycin are currently being tested concerning their effect (for potential use in valuable calves and in AIDS patients) (de Graaf *et al.* 1999b; Divers & Peek, 2008).

Results from Viu, Quilez, Sánchez-Acedo, del Cacho & López-Bernard (2000) showed that paramomycin, used prophylactically during 11 days (100 mg/kg BW daily), has positive effects by reducing oocyst excretion, as well as the frequency and duration of diarrhea in lambs and calves. Similar to what occurs with halofuginone, oocyst shedding resumes after the treatment period (Fayer & Ellis, 1993; Viu *et al.* 2000; Radostits *et al.* 2007). It is often used for the treatment of human cryptosporidiosis, but has doubtful results in calves (Constable *et al.* 2009).

Decoquinate had positive effects in kids and lambs but not in calves (de Graaf *et al.* 1999b, O'Handley & Olson, 2006). It is a hydroxyquinolone that exerts its effect by inhibiting the cytochrome-mediated electron transport in the mitochondria. Several studies showed no positive effects in the reduction of oocyst excretion or reduction of diarrhea, so it is not recommended for cases of cryptosporidiosis in cattle (Moore *et al.* 2003).

The macrolide azythromycin at a dose of 1500 mg/calf/day (1 – 2 g/calf orally daily during 7 days, 30 – 40 mg/kg daily) has positive effects on oocyst shedding, clinical signs, weight gain and the reduction of mortality in affected calves. The effects of this drug seem to be both antimicrobial and prokinetic (Elitok, Elitok & Pulat, 2005). The high costs and its current use in human respiratory disease are, however, major disadvantages for the current use of azythromycin in bovine medicine (Constable, 2009; Foster & Smith, 2009).

As *Cryptosporidium parvum* is resistant to antibiotics or standard coccidiostats, their use is not advised. An exception is a mixed infection with bacterial pathogens in which the administration of antibiotics is suggested (Divers & Peek, 2008).

Lasalocid, an ionophore coccidiostat, is effective against *C. parvum* but only if given in very high doses (100 mg/kg/day for 11 days (Kaufmann, 1996)), that would be toxic for the calf (de Graaf *et al.* 1999b; Divers & Peek, 2008). In addition, its usage is not allowed in the EU, as it is considered a growth promoter (Rommel *et al.* 2000). Other studies performed by

Göbel in 1987 showed positive results when administering a daily dose of 15 mg/kg to calves suffering from cryptosporidiosis (Constable, 2009).

Due to these difficulties and the secondary effects of available medication in Europe, as well as the self limiting nature of the infection, the therapy of *Cryptosporidium* cases is mainly non-specific and supportive, by the administration of fluids through oral (in light cases) or IV route (more severe cases of dehydration) (Rommel *et al*. 2000; Tzipori & Ward, 2002; Divers & Peek, 2008).

However, before talking in a more detailed way about the practice of fluid therapy in diarrheic calves, an overview about diarrhea should be made; especially because the fluid therapy is routinely performed in any case of diarrhea, regardless of the cause.

Physiopathology of diarrhea:

Diarrhea is not a disease, it is rather a sign that is common to many diseases (Michell *et al*. 1998; Kaske & Kunz, 2003).

In a normally functioning gastrointestinal tract, there is equilibrium between absorption and secretion in the intestine. When the secretion of fluids into the gut lumen is higher than the absorption, due to either an increased secretion rate or a decreased absorption rate, we are dealing with a case of diarrhea (Michell *et al.* 1998; Kaske & Kunz, 2003).

Dehydration caused by loss of water and electrolytes in the feces should be considered as an important consequence of diarrhea (Michell *et al.* 1998; Berchtold, 2009). Diarrhea might be, however, beneficial in some extent, as it fastens the elimination of toxins and pathogens, decreasing their possibility to contact with the gut epithelium (Michell *et al.* 1998).

New-born animals are especially sensitive to diarrhea, due to the fact that their body is not able to compensate the loss of high volumes of fluid. Even though calves have a higher relative blood volume than adult animals, the kidneys are not yet fully developed, which reduces their capacity of concentrating the urine. In addition, calves are not as effective in compensating a hypovolemic shock, due to the reduced cardiac stroke volume and elasticity of the vessels, and consequently the high heart rate (Kaske & Kunz, 2003; Koch 2004). In fact, fluid losses in calves with diarrhea can reach 13 to 18% of body weight per day (Koch, 2004; Bechtold, 2009).

Alongside with the dehydration, diarrhea originates major electrolyte losses in affected calves. Mainly sodium and potassium are excreted. Unfortunately their loss can be easily masked by hemoconcentration that accompanies dehydration (Michell *et al.* 1998; Koch, 2004; Berchtold, 2009).

Decreased serum concentrations of glucose, sodium and chloride are common findings in diarrheic calves (Koch, 2004; Berchtold, 2009).

Sodium levels are low due to the losses in the feces. Muscle weakness and mental depression are direct consequences (Radostits *et al.* 2007). The low sodium concentrations also have an additional impact on the dehydration, as they force the water from the extracellular to the intracellular compartment. As a result, the shock symptoms might be much more pronounced in calves with hyponatremia (Michell *et al.* 1998; Kaske & Kunz, 2003; Koch, 2004). On the other hand, hyponatremia can be a consequence of hypovolemia, as hypovolemia increases the retention of water rather than sodium and leads to dilution of the ECF (Michell *et al.* 1998).

In rare cases, hypernatremia can be seen. This electrolyte imbalance can be related to overfeeding of sodium containing oral rehydration fluids (especially if the concentration of sodium in the fluid is higher than 140 mEq/L) or if the calf has no access or refuses to drink water. This electrolyte imbalance is evidenced by myoclonias, trembling and weakness (Michell *et al.* 1998; Kaske & Kunz, 2003; Berchtold, 2009). The keeping of a low concentration of glucose in oral rehydration fluids is an important measure to avoid hypernatremia, as glucose helps the absorption of sodium and high concentrations of glucose may lead to osmotic diarrhea with loss of water and creation of hypernatremia (Michell *et al.* 1998; McClure, 2001).

Often, in diarrheic calves, potassium levels are low due to losses in the feces, reduced intake and also due to the activation of aldosterone which promotes sodium conservation and potassium excretion by acting on the kidneys and colon (Michell *et al.* 1998; Koch, 2004; Smith, 2009). As a result, calves may show muscle weakness and recumbency, depression, muscle tremor, cardiac arrhythmia and even coma (Radostits *et al.* 2007).

However, in acute cases, hyperkalemia (> 5 mmol/L) can be detected (Kaske & Kunz, 2003; Berchtold*,* 2009), as it will be described later on. Hyperkalemia is particularly dangerous and life threatening as the potassium influences the cardiac muscle cells (Kaske & Kunz, 2003).

Calcium is often low and magnesium concentrations vary greatly in diarrheic calves (Michell *et al.* 1998; Berchtold, 2009).

The mentioned electrolyte imbalances are rather stable and can remain even 10 days after the successful treatment of diarrhea in calves (Berchtold, 2009).

When calves have diarrhea, they will suffer from an extracellular hypo-osmotic dehydration. Extracellular fluid volume (plasma and interstitial fluid) is greatly reduced and intracellular fluid volume slightly increased. If the diarrhea is chronic or just before death, a hyperosmotic dehydration can occur (Koch, 2004; Berchtold, 2009).

A summary of the pathogenesis of dehydration is illustrated on the graphic nr. 1 in the attachments.

At the beginning of the dehydration a calf shows some degree of weakness, but it is still able to stand and suckle. In a more advanced stage, calves lose their ability to stand, attain a poor body condition and can even show signs of hypovolemic shock due to the high fluid

losses (Naylor *et al.* 1999; Kaske & Kunz, 2003; Berchtold, 2009). Shock is evidenced by lying on the side, tachycardia, cold extremities and comatous behavior (Naylor *et al.* 1999; Kaske *et al.* 2003).

In addition to the dehydration and loss of electrolytes, glucose concentration in the blood is reduced greatly (< 3,5 mmol/L), especially in the last hours before death (Kaske & Kunz, 2003; Koch, 2004). Hypothermia (temperature under 38,5ºC) is very commonly associated with dehydration and hypoglycemia. The lack of energy in form of glucose stimulates the brain to diminish the body temperature in order to save the remaining energy for the life supporting functions and increase in this way, the life time of the affected calf (Kaske & Kunz, 2003). It is hence, important to supply glucose before warming the calf with an exterior heat source (infrared) (Kaske & Kunz, 2003).

Metabolic acidosis:

An important consequence when we are dealing with diarrhea is metabolic acidosis (Naylor, Zello & Abeysekara, 2006; Berchtold, 2009; Lorenz, 2009).

While normal plasma pH in a calf ranges from 7,33 to 7,37 and concentration of HCO_3^- falls between 23 and 29 mmol/L, in a diarrheic calf these are usually much lower (Kaske & Kunz, 2003).

Metabolic acidosis in diarrhea seems to be associated to different factors:

- bicarbonate (HCO_3^-) ion loss in the diarrheic feces - substraction acidosis,
- reduced renal excretion of H^+ ions due to dehydration and consequent reduction in glomerular filtration rate – retention acidosis,
- organic acids in the plasma (increase in anaerobic glicolysis with formation of lactate due to loss of blood volume and lack of oxygen delivery) – addition acidosis (Kaske & Kunz, 2003; Koch, 2004; Smith, 2009; Lorenz, 2009).

In cases of diarrhea in calves, due to the villous atrophy and consequent malabsorption, intestinal bacteria ferment the nutrients and cause increase in D- and L-lactate (Lorenz, 2004; Berchtold, 2009; Lorenz, 2009; Smith, 2009). Both lactate isomers are absorbed in the gastrointestinal tract but their hepatic metabolism shows major differences. While L-lactate is easily metabolized, D-Lactate's metabolism is much slower, which suggests that metabolic acidosis is primarily linked to D-Lactate (and not as much to L-Lactate) increase (Kasari, 1999; Lorenz, 2004; Berchtold, 2009; Lorenz, 2009).

Although, clinical signs for metabolic acidosis are not specific, signs of depression of the central nervous system (due to the prompt penetration of D-Lactate into the CSF space and the direct neurotoxicity) seem highly associated with the degree of metabolic acidosis. These include weakness, ataxia, loss of suckle, menace, palpebral and panniculus reflexes and loss of standing ability (Naylor *et al.*1989; Koch, 2004; Lorenz, 2004; Naylor *et al.* 2006;

Berchtold, 2009; Lorenz, 2009). Especially the loss of the palpebral reflex seems associated to an increase in D-Lactate (Lorenz, 2004; Lorenz, 2009). The loss of the suckle reflex is most often caused by the combination of dehydration and metabolic acidosis. Its loss due to D-Lactate increase might be due to consequent loss of muscular tonicity and not because of the calf's inappetence (Koch, 2004; Lorenz, 2004; Lorenz, 2009).

The determination of D-lactate is only possible via liquid chromatography or measurement of enzymes, not with the portable lactate analyzers which only determine L-lactate concentration. So, as it is intricate to determine D-lactate, it is not usually done in practice. This fact is rather disadvantageous, as the D- isomer seems to be the main responsible for metabolic acidosis (Rollin *et al.* 2006; Lorenz, 2009).

The occurrence of acidosis in calves suffering from diarrhea is also directly related to an imbalance of the strong ions (Kasari, 1999; Kaske & Kunz, 2003; Constable, Stämpfli, Navetat, Berchtold & Schelcher, 2005).

Strong ions are characterized as nonbuffer ions, due to their completely dissociated status at physiologic pH, incapacity of taking part in chemical reactions and capacity of exerting an electrical effect (Smith, 2009).

Sodium and potassium are the main strong cations, some contribution arising from calcium and magnesium. Chloride, D- and L-Lactate and organic acids are the main strong anions.

According to the strong ion theory, the relationship between strong cations and anions determines greatly the acid-base status of an animal. In case of diarrhea, the great loss of cations (sodium and potassium) relative to normal or increased strong anion amount (mainly D-Lactate), originates a metabolic acidosis (Constable *et al.* 2005).

Strong ion difference can be calculated by the following, simplified formula:

$SID = [Na^+]+[K^+] – [Cl^-]$

In order to avoid metabolic acidosis, the sum of the cations (sodium and potassium) should exceed the strong anions (chloride). It should, therefore, have a minimum value of 60 to 80 mEq/L in diarrheic calves (Smith, 2009).

The severity of the acidosis can also be determined by the base excess (BE) as well as the base deficit (BD or negative base excess) values (Kasari, 1999; Nappert & Naylor, 2001; Koch, 2004).

The Base Deficit value (or negative base excess) is defined as "the amount of alkali required to restore one liter of blood to a pH of 7.4 at a pCO_2 of 40 mmHg" (Naylor *et al.* 2006 – from Ganong 1987). They represent the difference between the total concentration of buffer substances and their physiological concentration after blood gas analysis (Kaske *et al.* 2003). In a physiological case, both values are equal to zero, while in case of a metabolic acidosis, there is a negative base excess or a base deficit (Kasari, 1999).

Values ranging from -2 to 2 of the base excess are still considered normal, while lower values are considered metabolic acidosis and higher values represent a metabolic alkalosis (Kaske & Kunz, 2003; Radostits *et al.* 2007).

Even though there are ways of determining strong ion acidosis by laboratory tests (acid-base and blood gas analysis), determination of metabolic acidosis is still done routinely by the interpretation of clinical signs of affected calves (Nappert & Naylor, 2001; Rollin *et al.* 2006; Lorenz, 2009; Berchtold, 2009). However, often the clinical signs are not as accurate, and can mislead us in respect to the severity of acidosis (Lorenz, 2009).

A scoring system for the determination of the degree of metabolic acidosis was created and can be seen in the attachment (Table no. 2). Following this, scores are attributed to the different clinical signs and summed up. A maximum value of 15 was attributed to severely acidotic calves with dehydration (Kasari, 1999).

Relating the clinical signs to the base excess value, we can consider that if the calf is in sternal recumbency and just slightly weak and dehydrated, its base excess value should be between -6 and -12 mmol/L. If the calf is in lateral recumbency, showing signs of hypovolemic shock, base excess value is estimated to be between -12 and -20 mmol/L. Very bad prognosis is considered in cases with BE of -30 mmol/L (Kaske & Kunz, 2003).

One can say that at a blood pH of 7, the calve is in a critical health situation, a pH value under 6,8 causes usually the death of the calf (Kaske & Kunz, 2003).

In order to control the acidosis in the organism, there are several buffer systems that can be considered.

Bicarbonate is the buffer of choice for the treatment of metabolic acidosis (Kasari, 1999; Lorenz, 2009).

The bicarbonate- carbonic acid buffer system regulates the organism by the following formula: $CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows H^+ + HCO_3^-$

Administering bicarbonate, hence, moves the reaction to the left and helps the elimination of H^+ ions (Kasari, 1999).

The amount necessary to reverse the calf's acidosis can be determined by the following formula:

HCO_3^- needed (mmol) = BE (mmol/L) x Body Weight (kg) x 0.6

Being the value of 0.6 the distributing factor of bicarbonate in the calf's body.

As the molecular weight of sodium bicarbonate ($NaHCO_3$) is 84 g/mol. This value can be used to calculate the volume of a 8.4%, a 4.2% or a 1.26% solution necessary to balance out the bicarbonate deficiencies (Kaske & Kunz, 2003; Koch, 2004; Naylor *et al.* 2006; Lorenz, 2009).

For instance, a calve weighing 40 kg, with a BE of -20 mmol/L needs 480 mmol of $NaHCO_3$, or 480 ml of a 8.4% solution, 960 ml of a 4.2% solution or 3.2L of a 1.26% solution (Kaske & Kunz, 2003).

Evidence suggests that severity of metabolic acidosis varies according to the age of the calf. It is less severe in the first week of the calf's life and rises to a much higher level when it is older. In over 8 day-old calves, base deficit can reach twice as high values than in younger ones. Hence, older calves might need a higher supply of bicarbonate (Naylor *et al.* 1989; Kaske & Kunz, 2003; Koch, 2004; Berchtold, 2009; Lorenz, 2009).

Even though they often co-exist, dehydration and metabolic acidosis do not completely correlate. In this way, calves that are more dehydrated (more sunken eyeballs) may show less severe metabolic acidosis (Naylor *et al.* 1989; Lorenz, 2004; Berchtold, 2009). A study by Lorenz (2004) proved that finding by comparing the degree of acidosis with the degree of dehydration (estimated by the blood urea concentration).

Blume (2007) showed a direct relationship between hypothermia and acidosis. The lower the body temperature, the higher the degree of acidosis.

In 2002, Lorenz discovered that the base excess value is not completely correlated to the concentration of D-Lactate, varying independently. However, when the base excess value falls below -25 mmol/L, D-Lactate showed consistently high values (Lorenz, 2004).

In conclusion, metabolic acidosis is a frequent finding in cases of diarrhea. It is related to losses of bicarbonate, retention of hydrogen ions, formation of lactatic acid and other organic acids and strong ion imbalances.

Fluid Therapy:

The first step when starting the supplementation of fluids is the assessment of the degree of dehydration of the calf, usually by estimation of extracellular fluid loss in general physical examination (Constable, Walker, Morin & Foreman, 1998; Naylor *et al.* 2006; Smith, 2009).

Several attempts of determining the dehydration during the physical examination have been carried out. Constable *et al.* (1998) determined the methods that showed the most consistent and practicable results: degree of eyeball recession into the orbit (enophtalmus), skin fold duration on the neck, and determination of the concentration of protein in the plasma.

By everting the lower eyelid and evaluating the distance between eye and orbit the degree of enophtalmus can be assessed (Radostits *et al.* 2007; Smith, 2009). In the list of differential diagnosis cachexia (e.g. in chronic diarrhea) should also be considered, due to the consumption of body fat stores behind the eyeball (Smith, 2009).

The elasticity of the skin is evaluated by pinching a skinfold in the middle of the lateral cervical area, rotating it 90 degrees and, after releasing, measuring the time for the fold to disappear (Naylor *et al.* 2006; Radostits *et al.* 2007; Smith, 2009).

Eyeball recession and skin elasticity are accurate and easily accessed in physical examinations, but can be slightly subjective and require some degree of experience (Constable *et al.* 1998; Naylor *et al.* 2006; Smith, 2009).

The consistency of the feces can give some good indications about the severity and rate of fluid loss which can be helpful in the choice of the fluid therapy protocol (Kaske *et al.* 2003). However, fecal consistency is not a reliable measure to evaluate the improvement of dehydration (Brooks, Michell, Wagstaff & White, 1996a).

The rectal temperature can also be an indicator of the degree of dehydration as it is correlated with the reduction of cardiac output (Berchtold, 1999).

In either mechanism that leads to diarrhea (secretory, malabsorptive…), there is always an increased excretion of electrolytes and water in the feces. In response to this losses the calf suffers from dehydration, electrolyte imbalances (decreased sodium and increased or decreased potassium), metabolic acidosis, rise in the D-lactate concentration and an energy imbalance (due to the malabsorption of nutrients and the calves' anorexia) (Brooks *et al.* 1996a; Kaske & Kunz, 2003; Smith, 2009).

Nowadays, even with considerable advance in research, available solutions have still similar composition to the originally developed (Smith, 2009). Taking this into account, modern rehydration solutions should:

- Most importantly, reestablish the circulating blood volume and correct extracellular dehydration by supplying agents that favor water and sodium absorption in the intestine (like glucose, acetate, citrate, propionate, or glycine) (Brooks, White, Wagstaff & Michell, 1996b; Naylor *et al.* 1999; Berchtold, 1999; Berchtold, 2009).
- Correct electrolyte imbalances. Especially by providing an adequate amount of sodium to balance extracellular fluid volume (Brooks *et al.* 1996b; Michell *et al.* 1998; Berchtold, 1999; Constable, Thomas & Boisrame, 2001; Berchtold, 2009; Smith, 2009).
- Correct metabolic acidosis: by supplementing alkalinizing agents (bicarbonate, acetate or propionate) (Michell *et al.* 1998; Berchtold, 1999; Constable *et al.* 2001; Naylor *et al.* 2006; Berchtold, 2009; Smith, 2009) and by using electrolyte solution with a high strong ion difference. A decrease in D-lactate levels in the blood can also be attained simply by increasing perfusion and, hence, renal elimination (Berchtold, 2009).
- Supplement the calves with a sufficient amount of energy to correct the negative energy balance (Brooks *et al.* 1996b; Michell *et al.* 1998; Constable *et al.* 2001). Dextrose is to be used with caution, because even though it is an important source of energy it can decrease the voluntary milk intake and the serum phosphorus levels (Berchtold, 2009).

- Decrease mental depression and reestablish suckle reflex (directly related to the correction of acidosis together with the rehydration) (Kasari, 1999; Berchtold, 1999; Berchtold, 2009).
- Help in the repair of the intestinal mucosa (Berchtold, 1999; Naylor *et al.* 1999; Berchtold, 2009).

The daily fluid requirements can be calculated, taking the amounts for replacement, maintenance and current losses through diarrhea into account. Replacement fluid volume can be calculated by the formula: Replacement fluid = dehydration (%) x bodyweight (kg)

Maintenance fluid volume for a calf is about 80 to 100 mL/kg and current losses can reach values as high as 7 L/day, depending on the severity of the diarrhea (Garcia, 1999; Kaske & Kunz, 2003; Koch, 2004; Berchtold, 2009).

Whether we opt for oral or intravenous fluid therapy is dependent on the degree of dehydration of a calf, if it still can suckle or not, the degree of CNS depression, and its remaining strength (ability to stand) (Berchtold, 1999; Naylor *et al.* 2006; Berchtold, 2009).

On a overall basis, at the first signs of diarrhea, if the dehydration status is not very pronounced (under 8% dehydration) and the calf still stands and is able to suckle, oral rehydration therapy should be considered as a first step in the supportive therapy (Naylor *et al.* 2006; Smith, 2009; Berchtold, 2009). On the other hand, if the dehydration is more pronounced (> 8% dehydration) and the calf is unable to suckle, showing signs of CNS depression, intravenous fluid therapy is more indicated (Koch, 2004; Naylor *et al.* 2006; Berchtold, 2009).

Oral electrolyte solutions:

As oral rehydration fluids are cheap and easily used in farms, they are a treatment of choice for neonatal diarrhea (Kaske & Kunz, 2003; Smith, 2009). This method can be even applied by the farmer himself, without the help of the veterinarian (Kaske & Kunz, 2003).

They can be used in calves that show a suckle reflex, which normally indicates an, at least partially, functional gastrointestinal tract. Administration in case of a poorly functional digestive tract (e.g. Ileus) can result in complications such as bloat and rumen acidosis (Naylor *et al.* 1999; Naylor *et al.* 2006; Smith, 2009).

The administration should start as soon as the feces get a slightly thinner consistency and continued until the normal pasty consistency is recovered (Kaske & Kunz, 2003).

It is important to retain that even calves with profuse diarrhea, still have some degree of absorptive capacity in their intestines and should be fed with an increased fluid volume (McClure, 2001; Kaske & Kunz, 2003).

The three crucial points when choosing an oral rehydration solution are sodium replacement, bicarbonate concentration and tonicity (Michell *et al.* 1998).

Sodium concentration in oral rehydration solutions should be between 90 and 130 mmol/L (Brooks *et al.* 1996b; Michell *et al.* 1998; Smith, 2009). Lower concentrations may not be able to correct imbalances and dehydration, higher concentrations could result in hypernatremia or, due to elevated osmolality, cause delays in abomasal emptying rates and ileus, predisposing to several gastrointestinal disorders (Sen, Constable & Marshall, 2006).

Sodium transfer consists of a passive transfer and is usually connected to the absorption or secretion of other products, like glucose and amino acids, by co-transport on the apical membrane of the enterocytes (Brooks *et al.* 1996b; Constable *et al*. 2001; McClure, 2001; Smith, 2009). Volatile fatty acids, like acetate or propionate, may also help sodium absorption and rehydration, by a different mechanism (Smith, 2009). The addition of these substances to the rehydration solutions increases, hence, the absorption of sodium (Brooks *et al.* 1996b; Michell *et al.* 1998; Smith, 2009; Foster & Smith, 2009).

Glucose-to-sodium ratio should be between 1:1 and 3:1 in an adequate rehydration solution. Higher ratios could predispose to osmotic diarrhea and hypernatremia, whereas lower ratios would fail to stimulate sodium absorption to a sufficient level (Brooks *et al.* 1996b; Michell *et al.* 1998; Constable *et al.* 2001; McClure, 2001; Smith, 2009).

Chloride loss in diarrhea is not as exuberant as in the case of sodium. It should be included in the electrolyte solution in a concentration between 40 and 80 mEq/L. The best approach is to keep the concentration at the low end of the interval, in order to increase the SID (strong ion difference), necessary to correct metabolic acidosis (Koch, 2004; Naylor *et al.* 2006; Smith, 2009).

Because hypokalemia causes profound muscular weakness together with weak suckling reflex, oral rehydration fluids should contain potassium in adequate amounts (10 – 30 mmol/L) (Kaske & Kunz, 2003; Smith, 2009).

In general, dehydrated calves should be fed with rehydrating solution containing 100 – 150 mmol/L of Glucose (Kaske & Kunz, 2003).

Even though rehydration fluids containing mainly electrolytes supplement the calves well with sodium and are efficient in the alkalinization process, they are very energy deficient (Brooks *et al*. 1996b; Constable *et al.* 2001; McClure, 2001).

Hypertonic solutions should not exceed a certain osmolality as they can cause osmotic diarrhea and severe villus damage, in addition to a delayed abomasal emptying. They can even worsen the diarrhea in calves affected by enteropathogens, instead of supporting the animals (Brooks *et al.* 1996b; Michell *et al.* 1998; Kaske & Kunz, 2003; Smith, 2009). Also, as previously stated, the excessive concentration of glucose may dilute the extracellular space and predispose to the establishment of hypernatremia (Michell *et al.* 1998; McClure, 2001).

Hypertonic solution with a osmolality of 500 to 600 mOsm/L are the best solution in calves that are not receiving milk or milk replacer, because they offer much more energy than

isotonic solutions (Smith, 2009) while warranting fast abomasal emptying (Constable *et al.* 2001).

As previously mentioned, dehydration is usually accompanied by acidosis. Thus, the oral administration of alkalinizing solutions is crucial in diarrheic calves (Michell *et al.* 1998).

Traditionally, the only method was the addition of alkalinizing products (bicarbonate, acetate or propionate) to oral electrolyte rehydrations fluids. Nowadays, in addition to these, and due to the recent discoveries of strong ion importance, oral electrolyte solutions should also be able to balance these strong ions (Smith, 2009).

Alkalinizing agents such as bicarbonate, acetate and propionate are often used in rehydration solutions. All of them are effective in alkalinizing the blood pH of acidotic calves. Bicarbonate has the advantage of acting directly and faster by binding to H^+ ions and dissociating into H_2O and CO_2, whereas acetate and propionate have to be metabolized by the liver. This way, the first is prefered in extremely dehydrated calves (Garcia, 1999; Koch, 2004; Naylor *et al.* 2006; Smith, 2009).

Although acetate and propionate have the disadvantage of their action being slower and their metabolism originating hydrogen ions, they also have several advantages. Unlike bicarbonate, acetate and propionate:

- Facilitate the absorption of sodium and water,
- Release energy when metabolized,
- Do not increase abomasal pH. Thus they do not destroy the natural barrier to bacterial infections (e. g. *E. coli* and *Salmonella*) (Koch, 2004; Naylor *et al.* 2006),
- Allow normal milk clotting.

(Michell *et al.* 1998; Constable *et al.* 2001; Kaske & Kunz, 2003; Naylor *et al.* 2006; Smith, 2009).

Studies by Nappert & Spennick (2003) proved that bicarbonate has an inhibiting effect on milk clotting, thus reducing growth rates if fed together with the milk replacer. Even though this mechanism is not fully understood to this date, feeding bicarbonate together with milk or milk replacer, should be avoided as inhibition of clotting would fasten abomasal emptying and avoid sufficient absorption by the intestine (Michell *et al.* 1998; Naylor *et al.* 1999; Constable *et al.* 2001; Kaske & Kunz, 2003; Naylor *et al.* 2006). A solution for this problem would be leaving an interval of 2 to 4 hours between bicarbonate and milk / milk replacer administration, using low concentrations of bicarbonate (25 mmol/L) together with citrate (12 mmol/L) or using acetate or propionate, which do not inhibit clotting (Nappert & Spennick, 2003; Naylor *et al.* 2006; Smith, 2009).

So, considering the importance of strong ion difference described before, it is important to retain that in a rehydration solution both the presence of an alkalinizing agent (in a concentration of 50 to 80 mmol/L and preferably acetate or propionate) and a high SID (> 60

– 80 mEq/L) are important to balance metabolic acidosis. Products with a low SID and without an alkalinizing agent should be avoided (Kaske & Kunz, 2003; Smith, 2009).
There are several commercially available rehydration products which vary in terms of composition and applied price (some examples can be seen in the attached table no. 4). Manual mixtures should be avoided due to frequent dosage errors of the ingredients (Kaske & Kunz, 2003).

Administration of oral rehydration fluids:

The administration of oral electrolytes should be scheduled as an extra meal between the administration of milk. Alternatively, in case of lack of labor or time, electrolytes can be given together with the milk replacer (if they contain acetate or low bicarbonate concentration) or *ad libitum* during all day (Smith, 2009).
In general, the recommendation is that a calf should receive 8 litres of oral rehydration fluid in a 24 hour- interval, divided into several volumes of 2 litres (Naylor *et al.* 2006).
It is advisable to offer the fluids in a nipple bottle to allow the normal functioning of the esophageal groove and the bypassing of the fore stomachs, reaching directly the abomasum (Kaske & Kunz, 2003). This method of administration allows a better and faster absorption of the fluids (Naylor *et al.* 1999).
The use of an orogastric tube to administer fluids to inappetent calves that lost their ability to suckle is a risky method. Some risks include the accidental administration into the respiratory tract (with consequent pneumonia) or, due to the failure of the esophageal groove reflex, into the rumen (with consequent ruminal acidosis). In addition, its continuous use is rather complicated and labor intensive (Naylor *et al.* 1999; Kaske & Kunz, 2003).
It is advised to continue milk or milk replacer feeding during oral rehydration in calves with diarrhea. The withdrawal of milk or milk replacer, that was thought to have a positive effect on intestinal healing and avoiding of SIBO (Small Intestinal bacterial overgrowth) as well as in increasing the ingested amount of rehydration fluid, is nowadays thought to have no advantage and can even have deleterious effects (Brooks *et al.* 1996b; McClure, 2001; Kaske & Kunz, 2003; Hanie, 2005; Divers & Peek, 2008; Smith, 2009).
Whole milk or milk replacer should be offered at least twice a day. If the environmental conditions are harsh (e.g. in winter when new born calves are kept outside in shelters) milk or milk replacer should be fed at least three times a day to maintain the body condition and bypass the losses, maldigestion and malabsorption of nutrients caused by *C. parvum* diarrhea (Divers & Peek, 2008).
Only in exceptional cases, if the calf is unable to suckle, milk replacer can be discontinued for up to 12 hours (but never for a longer period) (Smith, 2009).

In summary, oral rehydration therapy is a routine therapeutical approach for diarrhea in calves, when the animals are still able to suckle. Many products can be chosen but the ideal one should: balance out the sodium concentration of the ECF, enhance sodium and water absorption from the intestine, treat metabolic acidosis and provide energy, without causing any negative effects (McClure, 2001; Smith, 2009).

Intravenous Fluid Therapy:

In short points, the most important indications for IV fluid therapy in neonatal calves are:

- Dehydration (loss of over 8% of the body weight – skin fold delayed for 6 seconds, eyes sunken 4 mm and dry mucous membranes (Berchtold, 1999; Kaske & Kunz, 2003));
- Depression, weakness, inability to stand, absence of suckle reflex or coma;
- Anorexia during a larger period of time than 24 hours;
- Hypothermia (< 38ºC);
- Profuse and watery diarrhea (quickly worsening dehydration) (Berchtold, 1999; Koch, 2004; Berchtold, 2009).

In general, it is possible to affirm that IV fluid therapy, in contrast to oral rehydration, acts much faster, restoring normal perfusion, oxygen delivery and removal of metabolic products; and can be used in depressed and even comatose animals in which oral rehydration would be insufficient or even impossible (Berchtold & Prechtl, 2003; Berchtold, 2009). When we start to rehydrate orally, the intravenous therapy should start as soon as the animal enters the decompensated stadium (loss of suckle reflex, of the ability to stand and coma) which represents the start of a hypovolemic shock (Kaske & Kunz, 2003).
Intravenous therapy is easily achieved in a clinic, however in on-farm practice its use is more complicated (Garcia, 1999; Berchtold, 2009).

Types of intravenous rehydration solutions:

It is important to take into account the type, amount, route and rate of IV infusions when considering the use of IV fluids in calves (Berchtold, 2009).
In the treatment of calf diarrhea, both crystalloid and colloid solutions are used.
Crystalloid solutions have the advantage of being distributed easily throughout all body fluid compartments. They contain mainly electrolytes (sodium, chloride, etc.) but also organic compounds (glucose, dextrose, lactate, etc.) (Berchtold, 2009).
Several types of crystalline solutions are routinely used, including isotonic and hypertonic saline (0,9% and 7,4% NaCl, respectively), isotonic and hypertonic sodium bicarbonate ($NaHCO_3$), acetated and lactated Ringer's Solution, hypertonic dextrose (Berchtold, 2009).

The amount of sodium that should be present in an isotonic IV rehydration solution should be at least 140 mEq/L, in order to maintain or increase the extracellular fluid volume (Berchtold, 2009). Sodium concentration should not exceed 150 mmol/L to avoid hypernatremia (Kaske & Kunz, 2003).

In severe hypoalbuminemia (e.g. prolonged anorexia), it is advisable to begin the treatment with a colloid or whole blood transfusion, as the administration of a rehydration fluid containing sodium may reduce plasma albumin and exacerbate edema (Kaske & Kunz, 2003; Radostits *et al.* 2007; Berchtold, 2009).

Colloids are mainly used in cases where plasma volume expansion is wished, as they are large molecules that are unable to pass the cell membranes.

Often used solutions are: Whole blood, separated blood elements, plasma and glucose polymers (dextran preparations (dextran-70), hydroxyethyl starch preparations (hetastarch and pentastarch)) (Berchtold, 2009).

A standart approach to dehydration in calves is the administration of 10 litres of a physiologic NaCl 0,9% solution, added by 5% glucose and buffer solutions (Kaske & Kunz, 2003; Radostits *et al.* 2007).

Hypertonic saline solution:

The use of hypertonic saline solution (7,2% and 7,4%) may be a good approach to dehydrated and weak calves, often enriched with 6% dextran. The plasma osmolarity rises exponencially and forces the fluid into the vessels by osmosis, as a result, the blood pressure is quickly increased. By causing the expansion of the blood volume and due to its positive inotropism, it improves cardiac output and the blood circulation (which are reduced in dehydrated calves). Moreover, it is thought to activate leucocytes, protect endothelial cells and improve renal function (increase of glomerular filtration rate due to the improved perfusion) (Rocha e Silva, 1997; Kaske & Kunz, 2003; Koch, 2004; Isetta, 2007).

It should be given at a rate of 4 ml/kg bodyweight in four minutes. If the fluid is administered slower no increase in plasma osmolarity is noted; and if it's administered much faster bradycardia can occur (Rocha e Silva, 1997; Kaske & Kunz, 2003; Koch, 2004). However, the effect of hypertonic saline is very brief and can only be maintained for a longer time if the calf is offered an amount of at least 3 liters of oral rehydration fluid (if they do not drink spontaneously a drencher should be used) (Rocha e Silva, 1997; Kaske & Kunz, 2003; Koch, 2004).

In a study by Koch (2004), the administration of hypertonic saline showed positive results in weak and inappetent calves, by showing great improvement of the standing ability and suckle reflex.

The need of ingestion of fluids after the infusion of hypertonic solutions is, though, a point which only allows its use in moderate dehydration cases where the calf is still able to suckle. Calves that lost their ability to suckle should rather be treated with dropwise continuous

infusion of isotonic saline together with sodium bicarbonate (as they are often severely acidotic) (Kaske & Kunz, 2003).

Even though hypertonic saline can be used to resuscitate dehydrated calves, it has no effect in solving metabolic acidosis and should be supplemented with alkalinizing solutions (Koch, 2004).

Alkalinizing solutions:

A standart approach to calves that suffer from severe diarrhea and acidosis remains the administration of 1 to 4 Litres of sodium bicarbonate in form of a 1,3% isotonic solution, with a strong ion difference of 155 mEq/L. The capacity of this compound in buffering hydrogen ions alongside with the increase of the strong ion difference turns it into a valuable and rapidly acting alkalinizing agent (Berchtold, 1999; Iwabuchi, Suzuki, Abe & Asano, 2003; Naylor *et al.* 2006; Berchtold, 2009).

In addition to the isotonic solutions, hypertonic solutions of sodium bicarbonate are available. The most often encountered solutions are those with a concentration of 4,2% (1000 mOsm/L) or 8,4% (2000 mOsm/L), which are usually mixed with a certain amount of isotonic saline to originate only slightly hypertonic solutions (Koch, 2004; Naylor *et al.* 2006; Berchtold, 2009).

A good approach to an acidotic calve is the administration of 1 or 2 bottles (250 ml) of a 8,4% solution mixed with 5 litres of saline in order to increase the metabolic pH (Berchtold, 2009).

Administering undiluted 8,4% hypertonic solution should be avoided due to the adverse effects in acidotic and dehydrated calves (hyperosmolality of the extracellular fluid, hypernatremia, hypocalcemia, and paradoxic intracellular and CSF acidosis). In cases of concurrent respiratory disease in calves, the use of hypertonic sodium bicarbonate solutions is of even greater risk as those calves might be unable to exhale the excessive amount of carbon dioxide produced in the reactions (Koch, 2004; Naylor *et al.* 2006).

Besides sodium bicarbonate, lactate and acetate are alkalinizing agents included in many polyionic intravenous solutions (like Ringer's solution). Not only used in intravenous but also, as previously refered, in oral rehydration solutions, their alkalinizing effect is efficient but not as fast as the one from sodium bicarbonate. The effect is mainly secondary, as one of their metabolic byproducts is bicarbonate (HCO_3^-) (Garcia, 1999; Naylor *et al.* 2006; Berchtold, 2009).

If we have to choose between acetated and lactated Ringer's solution, it is preferable to opt for the acetated. The reason behind is that lactated Ringer's solution is a mixture of L- and D-lactate. As D-lactate may be already increased systemically, the administration of lactated Ringer's solution could worsen the clinical signs in acidotic calves. Another reason is the faster metabolization of acetated Ringer's solution which allows a faster systemic alkalinization (Berchtold, 2009; Lorenz, 2009). On the other hand, acetated solution has a

disadvantage due to the presence of gluconate in its composition, which is not well metabolized by new born animals (Berchtold, 2009).
As a guide line, sodium bicarbonate is advised in worse cases of acidosis while the two types of Ringer's solution are more indicated in case of slight to medium degree acidosis (pH > 7.2 or base deficit < 10 mEq/L) (Koch, 2004; Berchtold, 2009).

Correct administration of IV fluid therapy:

If we want to administer fluids intravenously, the first step is to create a suitable and patent entry to the vein. The most important veins for catheterization in diarrheic calves are the jugular and the ear vein. Although the ear veins seem to be very small, catheterization is still possible even in highly dehydrated calves (Roussel *et al.* 1996; Garcia, 1999; Kaske & Kunz, 2003; Berchtold, 2009).
Ear vein catheters show advantages when compared to jugular catheters. In a study by Roussel, Talioferra, Navarre & Hooper (1996), they caused less cases of thrombophlebitis and did not have to be changed as frequently. This way, they are considered to be safe, easy to put and less expensive than the jugular vein catheters (Roussel *et al.* 1996; Berchtold, 2009).
When considering the administration of alkalinizing solutions, infusions through the ear vein showed less acid base fluctuations and lower incidence of alkalosis after administration (Blume, 2007).
Complications of venal catheterization include phlebitis, edema or hematoma. A good hygiene and assepsia are crucial to avoid any bacterial invasion.
Systemic infections, due to tromboflebitis (secondary to venous catheter placement) are demonstrated by fever, painful and warm swelling at the vein site, anorexia and depression. Even though this risk is lower in ear catheters, their flow rate is lower and they are more prone to occlude (Roussel *et al.* 1996; Berchtold, 2009).
To avoid the risk of thrombosis, jugular vein catheters should be changed every 2 days. Ear catheters can remain for a slightly longer period of time (Roussel *et al.* 1996).
In clinic conditions, where the calves remain restricted to the space in the pens and are controlled regularly by clinic staff, a continuous drip infusion through the ear vein is the most rewarding procedure. Under field conditions however, jugular vein catheterization with relatively quick administration of fluids is more commonly used as the supervision of the calves can not be guaranteed (Roussel *et al.* 1996; Berchtold, 1999; Berchtold & Prechtl, 2003; Berchtold, 2009).
The placement technique of ear vein and jugular catheters follows certain patterns and is described in greater detail in the attachments (Pages 108 and 109).

It is crucial to avoid stress of the calf at any moment, as stressful situations can cause collapse and death in the animals with an already weak circulatory system (Kasari, 1999).

Once the venal access is established, the correct way of administration and amount of fluid has to be determined.

Dehydration status is normally determined by clinical signs, as it can be seen on the table no. 3 in the attachments (Berchtold, 1999). If there is a need for intravenous fluid therapy, it should be administered, having as a goal the recovering of the suckle reflex so that further rehydration can be attained orally (Berchtold, 2009).

The next step will be to determine the necessary fluid volume to balance the losses. The daily fluid requirements are obtained by the sum of replacement, maintenance and current losses through diarrhea, as referred previously in the chapter about diarrhea (Garcia, 1999; Kaske & Kunz, 2003; Koch, 2004; Berchtold, 2009).

Buffer requirements are then determined by the formula:

Bicarbonate requirements [mEq] = body weight [kg] x base deficit [mEq/L] x 0.6 [L/kg], (Garcia, 1999; Koch, 2004; Naylor *et al.* 2006; Berchtold, 2009), already mentioned before in the chapter about metabolic acidosis.

When treating the calf with IV fluids, improvement should be tightly monitored. Signs of improvement include recovery of normal mental and hydration status, reappearance of the suckle reflex and standing up. Within 30 to 60 minutes the calf should urinate (Garcia, 1999; Berchtold, 1999; Divers & Peek, 2008; Berchtold, 2009). If they do not improve, other diseases, such as septicemia, omphalitis or pneumonia, can be present.

As an example, a standard therapy for dehydrated calves includes:

- 5 L of isotonic saline (0,9% NaCl) + 250 mL of 8,4% hypertonic sodium bicarbonate (250 mEq HCO_3^-)

If calves do not improve after this treatment, they are thought to have a more severe acidosis and should receive a higher amount of bicarbonate (up to 750 mL of a 8,4% sodium bicarbonate solution (750 mEq HCO_3^-) added to the 5 L of NaCl). If the dehydration is still not improving, a transfusion of whole blood (800 – 1000 mL) is the next step (Berchtold, 2009).

It is important to retain that the infusion in the ear vein should not exceed a maximum rate of 60 ml/kg body weight/ hour due to its small diameter and low resistance to high pressures (Kaske & Kunz, 2003). In general, a maximum fluid administration rate of 80 mL/kg/h shouldn't be exceeded, as an excessive infusion rate may give rise to signs of overhydration and hypertension (pulmonary edema); preferably a much lower infusion rate (30 to 40 mL/kg/h) should be considered (Kasari, 1999; Naylor *et al.* 2006; Berchtold, 2009). Administration can be fast in the first hours and should be reduced as soon as the major fluid requirements are covered (Naylor *et al.* 2006).

It is important to administer the fluids at body temperature to avoid cooling of the calves (Berchtold, 1999). Together with the infusion, it is advised to warm the calf with infrared light to avoid further loss of energy (Kaske & Kunz, 2003). The continuous milk feeding throughout the therapy should not be forgotten (Berchtold, 2009).
As a final note, after the first part of the treatment and when the calf regained its capacity to suckle, further treatment can be administered orally (Berchtold, 2009).

Systemic complications:

It is necessary to be aware that cases of diarrhea can originate secondary infection and septicemia (Kaske & Kunz, 2003). Especially calves, because they are born almost deprived of any immunity status, are more susceptible to septicemia. They are dependent on the passive transfer of immunity and lack an established intestinal flora in their first days of life that would protect them against the colonization by pathogenic bacteria (Fecteau, Smith & George, 2009).
Regardless of the cause of diarrhea, there is an increased risk for small intestinal bacterial overgrowth. Combined with the SIBO, alterations in the intestinal function, damage of the intestinal wall and increased incidence of bacteriemia and septicemia can be present (Kaske & Kunz, 2003; Constable, 2004; Constable, 2009).
The increased D-lactate that is often present in diarrheic calves is mainly the result of bacterial fermentation, which is greater in case of SIBO (Lorenz, 2004; Lorenz, 2009).
If the feces of affected calves contain traces of blood and fibrin, there is a strong indication of the damaging of the intestinal mucosa with possible penetration of bacteria through the gut barrier. These can cause systemic infection evidenced by initially high temperatures which fall quickly to hypothermic levels (Radostits *et al.* 2007) (in cases of diarrhea, high temperatures can be masked by hypothermia), bad general condition (weakness, dehydration), reddened mucous membranes, injected episcleral vessels, omphalitis, lung affections and swollen articulations (increased heart and breathing rate are not entirely reliable signs) (Kaske & Kunz, 2003, Fecteau *et al.* 2009). Tachycardia, dry and cool mucous membranes and prolonged capillary refill time are common additional findings (Radostits *et al.* 2007).
In cases of blood or fibrin in the feces, profuse diarrhea (connected with dysbacteriemia) or insufficient colostrum supply at birth it is therefore indicated to use an appropriate antibiosis to prevent septicemia (Kaske & Kunz, 2003).
Additional side treatment in cases of diarrhea should mainly avoid the appearance of bacteriemia or septicemia, reduce pain and stress, avoid negative energy balance and help intestinal healing by providing nutrients (Constable, 2009).

Antimicrobials:

The most frequently encountered bacterium in cases of septicemia is *Escherichia coli* (Hanie, 2005; Fecteau *et al*. 2009). However, other bacteria like *Salmonella, Campylobacter, Klebsiella* and *Staphylococcus* have been isolated (Fecteau *et al.* 2009).

The most suitable groups of antibiotics are bactericidal and should act mainly on gram-negative bacteria (especially *E. coli*) (Constable, 2004).

The treatment with antimicrobials should be, however, limited to cases of systemic illness associated with diarrhea (with inappetence, fever, depression, dehydration) or when blood or fibrin is found in the feces (Constable, 2004). Calves that show normal general condition (active, good feed intake, no fever or dehydration) and do not suffer from a concurrent disease (omphalitis or pneumonia) should not be treated with antibiotics (Constable, 2009). Their use in these cases can mask the real problem that caused the diarrhea, influence secretion and absorption in the gut and can cause bacterial resistance (Kaske & Kunz, 2003).

The most used antibiotics for cases of septicemia include:

- As a first choice: Parenteral amoxicillin or ampicillin (10 mg/kg IM BID), parenteral potentiated sulfonamides (25 mg/kg IV or IM SID), oral amoxicillin trihydrate (10 mg/kg BID) combined or not with clavulanate potassium (12.5 mg of combination/kg BID) (Kaske & Kunz, 2003; Constable, 2004; Constable, 2009).
- Second choice: Cephalosporins from the third and fourth generation with high stability to β- Lactamase (e.g. Ceftiofur or Cefquinome 1-2 mg/kg SID or BID) (Kaske & Kunz, 2003; Constable, 2009)
- And the last choice: Parenteral fluorquinolones (e. g. danofloxacin 6 mg/kg SC once or repeating after 48 hours) (Kaske *et al.* 2003; Constable, 2009). This should be only given to calves with severe illness who require fluid therapy and in countries where their use is allowed (Constable, 2004; Constable, 2009; Fecteau *et al.* 2009).

Gentamycin and florfenicol should be avoided (as they are not effective against the Gram negative agents of diarrhea and, in the case of florfenicol, only act for a short period of time) (Kaske & Kunz, 2003; Fecteau *et al.* 2009). Gentamycin, in addition, has a prolonged withdrawal time in production animals (Constable, 2004).

The same applies to the use of Aminoglycosides, which should be avoided as their oral administration is not effective (poor absorbance in the gastrointestinal tract following oral administration or high withdrawal times and nephrotoxicity in parenteral administrations) (Constable, 2004; Constable, 2009).

The dosage and administration interval, as well as the duration of the therapy of at least 3 days are important points to be followed strictly to guarantee a successful treatment (Constable, 2004).

Anti-infammatory drugs:

Anti-inflammatory drugs are highly recommended, especially in diarrhea caused by viruses and cryptosporidia, in which the diarrhea symptoms are partly due to prostaglandins and other inflammatory mediators (increased chloride and water secretion, dilation and increased permeability of gut blood vessels and increased pain) (Kaske & Kunz, 2003). Analgesia is therefore an important component of the ancillary treatment decreasing pain and abdominal cramping which accompany cases of diarrhea, as well as the inflammatory response in the gastrointestinal tract and the signs of septicemia (Constable, 2009).

As the inflammatory response can be beneficial to a certain degree to help the repairing processes of the body, its interruption can be non beneficial in some cases. Also, the potential of these drugs to cause gastro intestinal ulcers or to influence renal blood supply (decreasing glomerular filtration rate) are documented disadvantages and are especially harmful in dehydrated calves (Kaske & Kunz, 2003).

The least harmful anti-inflammatory drugs seem to be those that act against COX-2 preferentially, including meloxicam (Kaske & Kunz, 2003).

Meloxicam acts as an analgesic and anti-inflammatory agent and should be given as a single shot (0,5 mg/kg) simultaneously with the oral rehydration therapy and the specific treatments, to decrease the signs of pain and overall health condition (Philipp, 2003; Constable, 2009). Improvement of the rectal temperature, general condition, behavior, feed intake, dehydration, pain signs and fecal consistency was detected by Philipp (2003).

Flunixin meglumine is another widely used anti-inflammatory drug in cases of diarrhea. It should be given once at a dose of 2,2 mg/kg (Barnett, Sischo, Moore & Reynolds, 2003; Constable, 2009). Studies by Barnett *et al.* (2003) proved that flunixin meglumine has a beneficial effect in the improvement of the general health of the calves, in the reduction of morbid days and the duration of antimicrobial treatments.

Both meloxicam and flunixin meglumine should not be given more than 3 days in a row, to avoid the occurence of abomasal ulcerations (Constable, 2009). They have highly beneficial effects as analgesic, anti-inflammatory, antipyretic and antisecretory agents, and by affecting intestinal motility (Constable, 2009).

Corticosteroids should not be used as they suppress the immune system and also due to the fact that their concentration is already increased in diarrheic calves (Lopez, Phillips & Lewis, 1975).

Other analgetics such as aspirin, bismuth subsalicylate or ketoprofen are not recommended (Constable, 2009).

Other ancillary treatment:

The acidification of the gut lumen seems to be an effective approach to the treatment of diarrhea. The abomasal pH in the newborn calf is rather high (pH 4.4 – 7.2) compared to the pH at 5 days of age (pH 1.4 – 1.7) (Constable, 2009). The high pH in the first days has the advantage of allowing the passage of the immunoglobulins without degradation but, on the other hand, it reduces the natural resistance to the infection with pathogenic bacteria (like *E. coli* and *Salmonella* spp.). As these bacteria are highly sensitive to the variations of the pH, acidifying the gut lumen could have an advantageous effect on the prevention of bacterial gut colonization (Constable, 2009).

Probiotics (lyophilized or living, non pathogenic bacteria or yeasts) can be used to limit the multiplication of pathogenic organisms. They decrease the growth of the population of pathogenic microorganisms by acidifying the gut content, by competing with the pathogenic bacteria for substracts and addesion sites and/or by influencing the secretion of the gut mucosa.

Usually used prophylactically, they may show advantages over antibiotics because the latter remove all bacteria in the organisms without making a distinction between potentially harmful and useful microorganisms (Kaske & Kunz, 2003).

In a study by Foster, Glass, Courtney & Ward (2003), *Lactobacillus acidophilus, L. reuteri* and *Bifidobacterium longum* showed capacity to reduce viability of *Cryptosporidium* oocysts, possibly by the production of anti-microbial products.

In cases of septicemia, however, the use of antibiotics and not probiotics is strongly indicated. The advantage of probiotics in the treatment of diarrhea and acidosis is not completely confirmed yet and their use is rather restricted (Constable, 2009; Lorenz, 2009).

Homeopathic drugs did not show any positive results yet and are therefore not included in the treatment of choice for cryptosporidiosis (Verdier, Öhagen & Alenius, 2003; Constable, 2009).

In short terms, the most important ancillary treatment in diarrhea is: antimicrobial with predominantly Gram-negative spectrum, non-steroidal anti-inflammatory medication for a reduced time (flunixin meglumine or meloxicam are the best) and continued milk feeding, in addition to the specific treatment for the diarrhea causing agent (Constable, 2009). As refered previously, no completely reliable specific treatment for cryptosporidiosis is known to this date, being halofuginone lactate the only routinely used substance in Europe.

Calves with septicemia should, in addition, receive intravenous fluids, eventually plasma transfusion, oral or parenteral nutrition and oxygen; in combination with a good bedding and an appropriate temperature. The goal is to avoid the calf's death or one of the worst

complications of septicemia: supurative meningitis, with very poor prognosis (Fecteau *et al.* 2009).

Prophylaxia:

The control strategies that should be implemented are very similar to those used to control coccidiosis and are aimed especially to the reduction of the oocysts to which an animal can be exposed and to the increase of the calves' resistance to enteropathogens in general, including *Cryptosporidium parvum* (Harp & Goff, 1998; O'Handley & Olson, 2006).

Since the infection by this parasite is often a herd problem (Rommel *et al.* 2000), especially through reuse of contaminated calf pens, the hygiene and husbandry are important management purposes in the herd (Harp & Goff, 1998; Kaske & Kunz, 2003; Foster & Smith, 2009).

Affected animals with high excretion of oocysts should be immediately isolated from the flock to avoid further spreading of the disease to animals and humans (Kaufmann, 1996; de Graaf *et al.* 1999b; Saini *et al*. 2000; Bopp, 2003). Feces should be removed and the contaminated areas should be readily cleaned. After cleaning, the use of an appropriate disinfectant is advisable (e.g. 5% formalin) (Kaufmann, 1996), in view of the great resistance of the oocysts to a broad spectrum of disinfectants (Rommel *et al.* 2000).

The colostrum administration in sufficient quantity and as early as possible is important to assure the passive transfer of maternal antibodies that limit the multiplication of cryptosporidia (Harp & Goff, 1998; Rommel *et al.* 2000; O'Handley & Olson, 2006).

The introduction of dairy calves to beef herds should be avoided, especially during calving season (Harp & Goff, 1998; O'Handley & Olson, 2006).

Due to their resistance to a high number of disinfectants, unless they are applied in hazardously high concentrations, and their ubiquitous existence, it is impossible to protect calves completely from the exposure to oocysts (Harp & Goff, 1998; de Graaf *et al.* 1999b; O'Handley & Olson, 2006). The lack of hygiene already in the calving pen (so the calves get infected right away at birth) and the low efficacy of the colostral antibodies against cryptosporidium are additional risk factors (de Graaf *et al.* 1999b; O'Handley & Olson, 2006; Radostits *et al.* 2007; Foster & Smith, 2009).

The zoonotic potential of cryptosporidiosis requires the special attention of the staff involved in the handling of calves, especially in respect to hygiene. Hands and equipment should be cleaned and disinfected regularly, not only to assure employee safety and transmission to other animals but also to avoid public health concerns (Harp & Goff, 1998).

In order to increase the calves' immunity, administration of vitamin A, D_3 and E, selenium and iron can be beneficial (Kaske & Kunz, 2003).

Stress should be avoided at any time in a new born calf's life as it can rise the concentration of cortisol and catecholamines that are now thought to increase the susceptibility to disease (Kaske & Kunz, 2003; Cortese, 2009).

Cleaning and disinfection:

As it was already stated, the hygiene management of a farm is a crucial factor in the disease management and could even be considered to be the most important tool in the fight against cryptosporidiosis (especially because there is no known, completely effective specific therapy) (de Graaf *et al.* 1999b; Kaske & Kunz, 2003). Considerably higher risk of infection in environments with poor hygiene was detected in studies by Hamnes *et al.* (2006) and Muhid *et al.* (2011).

By warranting a hygienic environment, the exposure of the animals to the infective oocysts is greatly reduced, which contributes to a much lower transmission rate (Bopp, 2003; de Graaf *et al.* 2003; Radostits *et al.* 2007). Establishing plans for HACCP (Hazard Analysis Critical Control Points) and IPM (Integrated Pest Management) is also advised (Saini *et al*., 2000; Kaske & Kunz, 2003).

Especially before the entrance of new calves, a clean and aseptic environment is of crucial importance (Fonseca, 2000).

Stables and pens should be cleaned regularly with a high pressure washer (moist heat), followed by disinfection. There are several commercially available disinfectants, including formalin, aldehydes and acids (de Graaf *et al.* 1999b; Kaske & Kunz, 2003). The best disinfectants are 5 to 10% ammonia, 6% hydrogen peroxide, 70% commercial bleach or 10% formalin (Saini *et al.* 2000; Bopp, 2003).

This method should be only applied when the stable is empty as the pressure cleaning produces infective aerosols and the disinfectants can be harmful to the airways. After disinfection the stables should be left empty to allow the dissipation of the harmful vapours (Kaske & Kunz, 2003). Hence, the All-in all-out method is highly advised as it allows better cleaning and disinfection (Radostits *et al.* 2007).

Oocysts are known to remain viable during extended periods of time (months). In cool, damp environments, oocysts are thought to survive up to 18 months (Saini *et al.* 2000; Radostits *et al.* 2007).

Variations in temperature as well as desiccation can influence to some extend the survival of the oocysts and their infectivity (Fayer *et al*. 2000). For instance, oocysts survive more than 12 weeks at temperatures of – 4ºC and 4ºC, in soil, water and feces (Olson, Goh, Phillips, Guselle & McAllister, 1999). Temperatures of -70ºC inactivate the oocysts in 1 hour and -20ºC in 24 hours (Fayer *et al.* 1998). By heating the oocysts to 55ºC the loss of infectivity

takes place in 30 seconds, also at 60ºC for 15 seconds and at 70ºC for 5 seconds (Olson *et al.* 2004).

In addition to the temperature, oocysts show marked resistance to chlorination and silage processing (Olson *et al.* 2004).

Tiranti *et al.* (2011) showed that *cryptosporidium* oocysts survive best in poorly drained soils with a high degree of moisture. Effluents from the farms should be tightly controlled in order to avoid spreading of the oocysts or the contamination of the water supplies (Bopp, 2003; Olson *et al.* 2004).

In addition to the cleaning and disinfection, the pest control has to be taken into account due to their role as transmitters and transporters of the infectious oocysts (Kaske & Kunz, 2003; Radostits *et al.* 2007).

Vaccination:

Before speaking about the approaches of vaccination against cryptosporidiosis, it is of some importance to focus upon the natural host immunity against cryptosporidiosis (Laurent *et al.* 1999; Gookin *et al.* 2002).

Immunity against cryptosporidiosis focuses upon innate and acquired immune responses. Innate responses include inflammatory cell infiltrations (macrophages and neutrophyls) and the secretion of immune molecules (prostaglandins, interleukins and other factors).

Humoral immunity by antibodies with origin in B-cells seems to play a quite reduced role in solving *Cryptosporidium* infection. However, both serum and secretory antibodies are produced in a *Cryptosporidium* infection (Laurent *et al.* 1999; de Graaf, Spano, Petry, Sagodira & Bonnin, 1999a; Gookin *et al.* 2002), antibodies may only be important in the prevention of reinfection (Tzipori & Ward, 2002).

The T-cells, on the other hand, are considered to be an important factor in the fight against cryptosporidiosis. In fact, CD4+ T-Cells have shown to be the most important host defence against this parasite (de Graaf *et al.* 1999a; Laurent *et al.* 1999; Gookin *et al.* 2002). The cytokines produced by the Th1 CD4+ cells, namely IFN γ and IL-2, are, together with the T CD4+ cells itself, important. They promote the cell mediated immunity against *Cryptosporidium* by stimulating phagocytes and cytotoxic T-cells (CD8 +) (de Graaf *et al.* 1999a; Gookin *et al.* 2002).

In fact, in a study conducted by Fayer *et al.* (1998), results showed that both CD4+ and CD8+ lymphocytes were increased, which supports the previous statement about the importance of acquired cellular immunity in the host defense against cryptosporidiosis.

Vaccination of cows for the prevention of diarrhea of the offspring focuses mainly on diarrhea caused by *E. coli, Rotavirus* and *Coronavirus*. The basis of maternal vaccination is the

passage of the antibodies, formed after vaccination during the dry period, to the colostrum, allowing the passive transfer to the calves. To be effective, the colostrum and milk should be administered to the calf during 10 days (to allow the local immunity formation after the closure of the gut barrier) (Kaske & Kunz, 2003).

A vaccine for the immunization of cows, to reduce incidence and severity of cryptosporidiosis, by enhancing passive transfer of maternal antibodies in calves, is planned for the near future (Divers & Peek, 2008). The main target groups for the vaccine would be neonatal calves and children from the developing world (deGraaf *et al.* 1999a).

As previously pointed out, the immunity against cryptosporidiosis seems to be mainly from the cellular type, possibly due to the parasite location inside the cell (Kaske & Kunz, 2003; Olson *et al.* 2004).

Vaccines have been already employed in several trials, for both passive and active immunization of calves. Although none of the vaccines was able to prevent the infection with *C. parvum*, in some cases reduction of clinical signs and oocyst excretion was observed following vaccination (de Graaf *et al*. 1999a; Olson *et al.* 2004). Passive immunization of dams followed by colostrum ingestion by the calves showed better results than the direct active vaccination of calves. Possible reasons might be the interference between colostral immunity and the vaccine in the first days of life or the late action of the vaccine (*C. parvum* infects calves in their first days of life) (deGraaf *et al.* 1999a).

Vaccines of whole organism or recombinant proteins administered to dry cows or oral vaccines given to calves at birth, prior to the ingestion of colostrum, reduced clinical signs and shedding at experimental conditions but were not successful in field trials (Harp & Goff, 1998; Perryman, Kapil, Jones & Hunt, 1998; Foster & Smith, 2009).

Studies by Perryman *et al.* (1998) were carried out by administering purified recombinant *Cryptosporidium* antigen to the dams. The colostrum obtained and administered to the calves caused a marked decrease in the clinical signs of cryptosporidiosis (no diarrhea) and the amount of shed oocysts was reduced.

Active vaccination was tested by Harp and Goff (1995) by giving an oral vaccine with lyophilized *C. parvum* oocysts to the calves after birth. Following this, diarrhea and oocyst shedding was shortened substancially. Field tests, however, did not show any positive results (Harp & Goff, 1998; deGraaf *et al.* 1999a).

The use of vaccines might be a good and important approach in the control of cryptosporidiosis. However, further studies are still necessary to be carried out to warrant success.

The use of vaccines as well as prophylactic chemotherapy against cryptosporidiosis is, nevertheless, questionable, to the point that this disease is usually mild and self-limiting and is followed by the development of immunity in the previously affected animals. Also as the

disease does not have long term effects on the production of ruminants the costs may not justify their use (deGraaf *et al.* 1999a).

The correct colostrum administration:

In order to fight the infective pressure in the environment, a calf should receive a sufficient quantity of colustrum as early as possible (Donovan, Dohoo, Montgomery & Bennett, 1998; Harp & Goff, 1998; de Graaf *et al.* 1999b; Kaske & Kunz, 2003; Hanie, 2005; O'Handley & Olson, 2006). This is so important because the bovine syndesmochorial placenta (*Placenta multiplex cotyledonaria*) doesn't allow the passage of immune globulins and the calves are born agammaglobulinemic, i.e. completely unprotected. They, hence, need to receive the necessary immune globulins through the ingestion of colostrum (Götze *et al.* 1993, de Graaf *et al.* 1999a). Weak calves that are unable to suckle should be supplied with colostrum via an orogastric probe (Kaske & Kunz, 2003; Hanie, 2005; Radostits *et al.* 2007).

The supplied colostrum should be clean, undiluted, given at body temperature and originated from mastitis free cows. Its supply should start already at the first hour of birth, when the absorption of immune globulins in the gastrointestinal tract is still very high, and continued during the next 24 hours, during which the absorption is gradually decreasing (Kaske & Kunz, 2003; Hanie, 2005; Radostits *et al.* 2007).

Ideally, calves should ingest 80 to 150g of colostral immunoglobulin G1 in the first hours after birth (Radostits *et al.* 2007). A good measure is, hence, the ingestion of 4 litres of colostrum in the first 12 hours (2 litres already in the first hour of life) (Kaske & Kunz, 2003; Hanie, 2005; Radostits *et al.* 2007), or, preferably, 4 litres of colostrum in the first 6 hours after birth (G. Stilwell, personal communication on the 22nd Nov. 2011). Beef calves should be observed to confirm correct nursing behavior (Hanie, 2005).

The ingestion of colostrum provides the calf with essential maternal antibodies (especially IgG_1, but also IgM and IgA), maternal lymphoid cells and immunoregulatory cytokines, which are essential for the development of the calf's immunity (Donovan *et al.* 1998; de Graaf *et al.* 1999a; Radostits *et al.* 2007). By the use of a colostrometer the specific gravity should be above 1.050 to guarantee an adequate IgG level in the colostrum (~50 g/L) (Hanie, 2005).

However, studies by Blume (2007) evidenced that colostrum administration is not enough to completely prevent the occurrence of diarrhea, as the studied calves all received sufficient quantity and good quality colostrum but showed diarrhea signs.

Colostrum not only provides the calf with humoral immunity but also is an important source of energy and nutrients and fastens the release of the meconium (Donovan *et al*. 1998; Kaske & Kunz, 2003).

To evaluate if a calve ingested a sufficient amount of colostrum blood or plasma can be tested either through determination of serum protein, of γ- Glutamil transferase (GGT), by the

sodium sulfate or the zinc sulfate test, the glutardialdehyde test or even by ELISA (Kaske & Kunz, 2003; Radostits *et al.* 2007). Even though it is usually only done in valuable calves, radial immunodiffusion, refractometry or latex agglutination at 24 hours of age are possible tests, being 1000 mg/dL serum IgG an adequate level (Hanie, 2005).

Preparing the cow for birth:

It is a fact that an easy birth originates stronger and more vital calves that are less prone to diseases. Hence, a cow should have a body condition score in a certain range (ideally 3,5 (± 0,25)) to allow an easy birth. To attain the desired score, the feeding of the cow prior to birth should be adapted reducing the energy content (Kaske & Kunz, 2003). Sufficient protein should be supplied to the dam before calving to guarantee vitality of the calf (Bopp, 2003).
Before calving, the cow should be introduced into a clean and sufficiently big calving pen (at least 10 m^2 for each animal). Ideally this area should be individual and be located in an area which allows easy control by the farmer (Kaske & Kunz, 2003).

Housing:

The keeping conditions influence greatly the health status of the calves and should be tightly monitored. As the full immunity is only developed at 3 to 4 months of age, at the beginning of life they should be kept separated from other calves or older animals, in individual pens (Kaske & Kunz, 2003; Radostits *et al.* 2007).
Contact with other animals increases the risk of infection, as it was studied by Muhid *et al.* (2011). However, in a study conducted by Kváč *et al.* (2011) infections with *C. parvum* were controversially more often detected in calves housed individually.
The outdoor keeping is preferred to the indoor keeping. As the bovine species lacks sweat glands, they tolerate low temperatures to a much higher extent than high ones. Their lower limit of the thermoneutral zone is at 14ºC (Kaske & Kunz, 2003).
It is of great importance to provide a dry (60 – 80% Humidity) and wind free environment to the calves, with a low concentration of harmful substances (Kaske & Kunz, 2003).

Cryptosporidiosis as a Zoonosis:

Cryptosporidium spp. is considered a ubiquitous, zoonotic, non host-specific parasite which can infect humans with an infective dose of only 10 oocysts (Tzipori & Ward, 2002).
Genetic analysis (PCR) revealed the presence of 2 genotypes of *C. parvum*: a genotype that is only present in humans (genotype 1 – considered *C. hominis*) and a genotype that can be present in a broad range of hosts and can circulate between humans and ruminants

(genotype 2 – *C. parvum*). If genotype 1 is isolated from a human being, the infection was originated after human-to-human transmission; on the contrary, if genotype 2 is isolated, the source of the infective oocysts can be human or originated from an animal. The latter facts can be helpful in the exclusion or confirmation of a zoonotic case (Saini *et al.* 2000; Tzipori & Ward, 2002; Olson *et al.* 2004; Radostits *et al.* 2007; Divers & Peek, 2008; O'Hara & Chen, 2011).

Taking the previous statements into account, humans can be infected by the zoonotic *Cryptosporidium parvum,* as well as the anthroponotic *C. hominis*. The differentiation can only be attained by molecular analysis, as these two parasites have a similar morphology. Important to refer is that *C. andersoni*, which often affects the bovine species, has no zoonotic potential (Olson *et al.* 2004; Mendonça *et al.* 2007).

Cryptosporidium parvum is considered an important agent of waterborne gastrointestinal disease in humans (Saini *et al.* 2000; Tzipori & Ward, 2002; O'Handley & Olson, 2006). Even though ruminants are thought to play an important role as reservoir hosts and transmitters of these parasites to humans, this risk is much less significant than once believed and is quite easily managed. By the way, many of the disease outbreaks in humans related to a ruminant reservoir are now shown to be of human origin (Olson *et al.* 2004; O'Handley & Olson, 2006; Xiao *et al.* 2001).

In fact, as suggested by Fonseca's (2000) results, the possibility of transmission of the zoonotic *C. parvum* from animal to human exists, however, as in the analyzed bovine samples no human type genotype (*C. hominis*) was detected, the transmission from humans to animals seems to be more restricted. These findings suggest that human cryptosporidiosis is not always a zoonosis.

As mentioned earlier, from all the Cryptosporidia species, *C. parvum* is the most important and considered the only species with zoonotic potential (Santín *et al.* 2004; O'Handley & Olson, 2006). However, doubts arose with the successful detection of *Cryptosporidium maleagridis* in humans with diarrhea, suggesting that it can as well be a cause of human cryptosporidiosis. *C. felis* and *C. canis* were sporadically detected in humans, but are not considered to be an important source for human infection (Pedraza-Díaz, Amar, Iversen, Stanley & McLauchlin, 2001; Xiao *et al.* 2001; Tzipori & Ward, 2002; Thompson *et al.* 2008).

Epidemiologic studies of the parasite propose that only young ruminants, especially neonatal calves, constitute a risk for zoonotic transmission. Calves older than 30 days are rarely infected by *C. parvum,* as well as older lambs and adult sheep. *C. andersoni* and *C. bovis* present in older cattle have no zoonotic potential (Santín *et al.* 2004; O'Hanley & Olson, 2006; Mendonça *et al.* 2007).

Cryptosporidiosis is considered an anthropozoonosis, especially immune compromised humans being sensible to this disease. The water of the areas in which cows are held can be

contaminated by oocysts and constitutes a potential risk of infection for humans (Mendonça *et al.* 2007; Divers & Peek, 2008).

Humans can be infected directly when they contact with infected humans or animals or by an indirect way through contaminated water or food (Fayer *et al.* 2000; Tzipori & Ward, 2002; Mendonça *et al.* 2007).

Usually the infection is self-limiting in otherwise healthy individuals and resolves after 9 to 15 days. Symptoms (which usually appear after an incubation period of 14 days, in immune compromised hosts) can be absent or characterized by a watery, profuse and mucus-containing diarrhea (rarely with traces of blood). Other signs such as nausea, vomiting, cramp-like abdominal pain and mild fever can occur (Laurent *et al.* 1999; Saini *et al.* 2000; O'Hara & Chen, 2011). In severe cases, malabsoption and wasting syndrome can be present, especially in AIDS patients. If the parasite colonizes the bile ducts, jaundice and pancreatitis symptoms can be present (Laurent *et al.* 1999).

Even though in cattle especially neonates and young animals are affected, in humans this fact is less reliable. All people involved in the handling of infected calves and their surroundings (technicians, farmers, veterinarians, students, etc.) are in potential risk of contracting the disease, especially if the hygienic and disinfection measures are deficient (Tzipori & Ward, 2002; Divers & Peek, 2008). However, children, pregnant women, elderly, malnourished and immune suppressed people are especially susceptible and more prone to develop severe clinical signs (Fayer *et al.* 1998; Saini *et al.* 2000).

Humans suffering from AIDS can be easily infected with *Cryptosporidium* and may suffer quite severe consequences (Saini *et al.* 2000; Tzipori & Ward, 2002). In fact, cryptosporidiosis is seen as one of the most severe and significant complications in cases of AIDS. If the CD4 T-cell number falls below 150/ml, exposure to *Cryptosporidium* originates severe, persistent diarrhea that can be life-threatening (Tzipori & Ward, 2002). The villi get atrophic, crypts hyperplasic and the lamina propria gets infiltrated by inflammatory cells (Chen *et al.* 2001).

As a further complication, cryptosporidiosis can give rise to cholangiohepatitis, cholecystitis, choledochitis or pancreatitis, being the main extraintestinal location of the parasite, the biliary tract (Tzipori & Ward, 2002; O'Hara & Chen, 2011). In chronic cases there can occur disruptions of the mucosal surface, fibrosis, cellular infiltrations and crypt abcessation. All these findings show the severity of the infection and the difficulty of controlling it in immune compromised individuals (Chen *et al.* 2001; Tzipori & Ward, 2002). A pulmonary form of cryptosporidiosis can occur especially in immune compromised individuals, due to the inhalation of the oocysts (Tzipori & Ward, 2002).

In individuals with AIDS, therapy approaches focus mainly on the reduction of the viral count and the increase in the concentration of CD4 lymphocytes by antiretroviral therapy. Paromomycin and nitazoxanide have been partly successful in the reduction of

cryptosporidiosis signs in humans affected with AIDS (Tzipori & Ward, 2002). As a matter of prevention, boiling the drinking water or using a filtering system (e.g. 1-µm pore filters) is advised in humans suffering from AIDS (Saini *et al.* 2000).

In general, it is possible to say that human cryptosporidiosis is mainly an issue in third world countries, as the incidence of AIDS and subnutrition, are much higher than in developed countries (O'Hara & Chen, 2011).

Other affected species:

All domestic mammals can be infected by *Cryptosporidium parvum*, and even cases in elk, deer and buffalo have been reported (Tzipori & Ward, 2002; O'Handley & Olson, 2006).

Small ruminants, lambs and kids, can be affected by *Cryptosporidium*. Lambs usually get infected at 4 to 10 days of age and kids at 5 to 21 days of age (Radostits *et al.* 2007).

The course of the disease is very similar to the one in calves, being the prepatent period for lambs around 2 to 7 days and for kids around 4 days. During excretion, small ruminants can even excrete a higher concentration in oocysts than calves (10^{10} oocysts per gram of feces). Symptoms of diarrhea usually last for 3 to 5 days. Simultaneous bacterial and viral infections are, as they are in calves, frequent but in small ruminants these seem to be less important for the expression of cryptosporidiosis (Koudela & Jiři, 1997; de Graaf *et al.* 1999b). Mortality due to cryptosporidiosis in small ruminants is higher than in calves and morbility often reaches 100% (deGraaf *et al.* 1999a).

Foals are often affected by *C. parvum*, however, it is not sure if it causes diarrhea. Usually the excretion of oocysts in affected animals occurs at an age of 4-19 weeks for up to 14 weeks, highest excretion rates occurring at 5 to 8 weeks of age (Rommel *et al.* 2000; Radostits *et al.* 2007). The animals are usually assymptomatic if their immunity is good. Immune deficient arabian and thoroughbred foals can show quite severe signs of disease (Fayer *et al.* 1998; Radostits *et al.* 2007).

Pigs are also often affected, especially at 1 – 6 months of age (Rommel *et al.* 2000). However, diarrhea is only present in piglets aged 2 weeks and younger (de Graaf *et al.* 1999b). In general, prevalence was discovered to be low in nursing piglets, occuring especially after weaning (Quilez, Sánchez-Acedo, Clavel, del Cacho & López-Berned, 1996). The parasite undergoes an incubation period of 4 – 5 days, after which the affected animals may show diarrhea for 2 – 3 days. The infection is usually subclinical, without a complete correlation between the detection of oocysts in the feces and the occurrence of diarrhea (Quilez *et al.* 1996; de Graaf *et al.* 1999b; Rommel *et al.* 2000).

The prevalence of cryptosporidiosis in dogs and cats can reach 20% and affects animals of every age group. The involved species of cryptosporidium are usually *C. parvum* and more specified species (*C. canis* and *C. felis*). After infection and the incubation time of about 2 to

14 days, the animals excrete the parasite for up to 80 days, which makes them an important source of human infection (AIDS patients) (Rommel *et al.* 2000; Thompson *et al.* 2008). There is not necessarily a correlation between the infection with cryptosporidia and the appearance of diarrhea (Rommel *et al.* 2000).

In 4 to 8 week-old rabbits, *Cryptosporidium parvum* can sporadically occur. Usually pre-weaned rabbits aged 8 to 12 days are affected and show diarrhea, anorexia, lethargy and dehydration (de Graaf *et al.* 1999b; Rommel *et al.* 2000).

In birds, turkeys may be infected by *Cryptosporidium meleagridis*. Especially the intestinal tract, the Fabricius bursa and the cloaca are affected. Diarrhea is the main symptom and mortality is moderate. Chicken can get infected by *Cryptosporidium baileyi* which usually affects the respiratory tract (larynx, trachea, bronchi and air sacs), the Fabricius bursa and the cloaca. Clinical signs include depression, anorexia, emaciation, coughing, sneezing and dyspnoea, rarely intestinal or renal derived symptoms can occur (de Graaf *et al.* 1999b).

Avian cryptosporidiosis occurs worldwide with prevalences ranging from 10 to 60%, and is responsible for high number of slaughter house rejections (mainly *C. baileyi* with airsacculitis) and, consequently, high economic losses (de Graaf *et al.* 1999b).

Case study in Hessen, Germany:

Introduction:

During our stay in the Clinic for Obstetrics, Ginecology, Andrology with veterinary ambulance service from the Justus Liebig University Giessen (Klinik für Geburtshilfe, Gynäkologie und Andrologie der Gross- und Kleintiere mit Tierärztlicher Ambulanz der Justus Liebig Universität Giessen), an analysis of the hospitalized calves was made.
The purpose was to study the number, prevalence, clinical signs, treatment and outcome of the calves infected with cryptosporidiosis.

Purposes:

- The determination of the number of calves suffering from diarrhea and of calves positive to cryptosporisiosis.
- The study of the clinical signs and the evolution of the affected calves.
- Description of the routine treatment employed in the clinic.

For this purpose, we examined a total number of 35 calves during our stay, recording the major health issues. The calves with diarrhea were submitted to a test to determine the presence of *Cryptosporidium* spp. oocysts.
In scope of this dissertation, a more profound observation of the calves suffering from cryptosporidiosis was made. Especially concerning their clinical signs, treatment and outcome.

Materials and Methods:

A total of 35 calves were examined, with ages ranging from a few hours to 3 weeks.
In cooperation with the clinic staff (veterinarians, technicians and students) a general physical examination was performed and specific diagnostic and treatment methods for each case were established.
The same way as it was routinely done in all animals of the clinic on a daily basis, a complete physical examination was performed in the 35 calves included in the study. The analysed parameters included: overall appearance; rectal temperature; aspect of the mucosa; hydration status; heart rate, rhythm and presence of abnormal sounds; respiratory rate, abnormal respiratory sounds, signs of respiratory disease (coughing, nasal discharge…); umbilicus (size, temperature, humidity, consistency and presence of hernia); aspect of the articulations; head reflexes; fecal consistency; abdominal tension. However, the complete description of all these signs is beyond the scope of this dissertation. Especially calves

suffering from diarrhea were weighed at the day of entrance in the clinic and several times throughout their stay.

As a routine approach, all calves were fed with milk replacer (Milli® from Sano), 3 times a day. Hay and water were offered *ad libitum*.

Oral rehydration fluid with balanced electrolytes was offered *ad libitum* through a nipple bucket between the milk replacer to calves suffering from diarrhea. Acid-base correction was not attempted by this fluid as it was usually corrected by IV or separate oral administration of sodium bicarbonate.

Fig. 12: Housing of the calves suffering from diarrhea (Original picture).

The calves suffering from diarrhea were housed separately in an area of the clinic called “Infective stable” and in individual pens covered in tiles and on a straw bed. At the entrance of this area a disinfecting pediluvium was installed. Special aprons and personal protection were used, followed by personal disinfection upon leaving the infective area.

Desinfection of the pens was attained by application of VENNO® VET 1 super and NEOPREDISAN® 135-1 (Menno Chemie Norderstedt). A thorough cleaning and disinfection were carried out by the clinic assistants upon leaving and before the entrance of new patients.

Feces were taken from the calves suffering from diarrhea in sterile plastic containers and submitted to the clinic’s laboratory in order to search for *Cryptosporidium* spp. oocysts. The analysis was done with the assistance of the laboratory technicians.

The procedure included the following steps:

1. Feces were collected in clean plastic containers and analyzed immediately after collection or after a short storage in the refrigerator (4 – 5 ºC).

2. In the laboratory, fecal containers were opened and a small quantity of diarrheic feces placed on a degreased microscope slide.

3. An approximately equal quantity of carbol fuchsin coloring was added.

4. With another degreased microscope slide, the mixture was spread over both microscopic slides and left to dry.

5. When almost dry, a drop of immersion oil and a cover slip were put on top.

6. The slides were observed under a light microscope with the 40 x objective (eventually also the 100 x).

7. Determination of the severity of infection: 1 – 2 oocysts/ microscopic field: +; 3 – 7 oocysts/ microscopic field: ++; 8 – 12 oocysts/ microscopic field: +++.

Calves suffering from cryptosporidiosis were then observed in a more detailed way and once a day during the next days, recording their clinical signs, treatment and outcome.

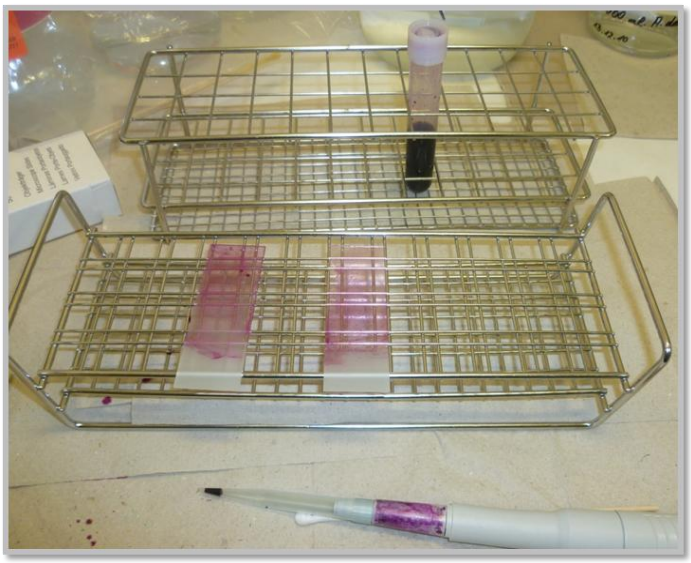


Fig. 13: Carbolfuchsin staining of the fecal samples (Original picture).

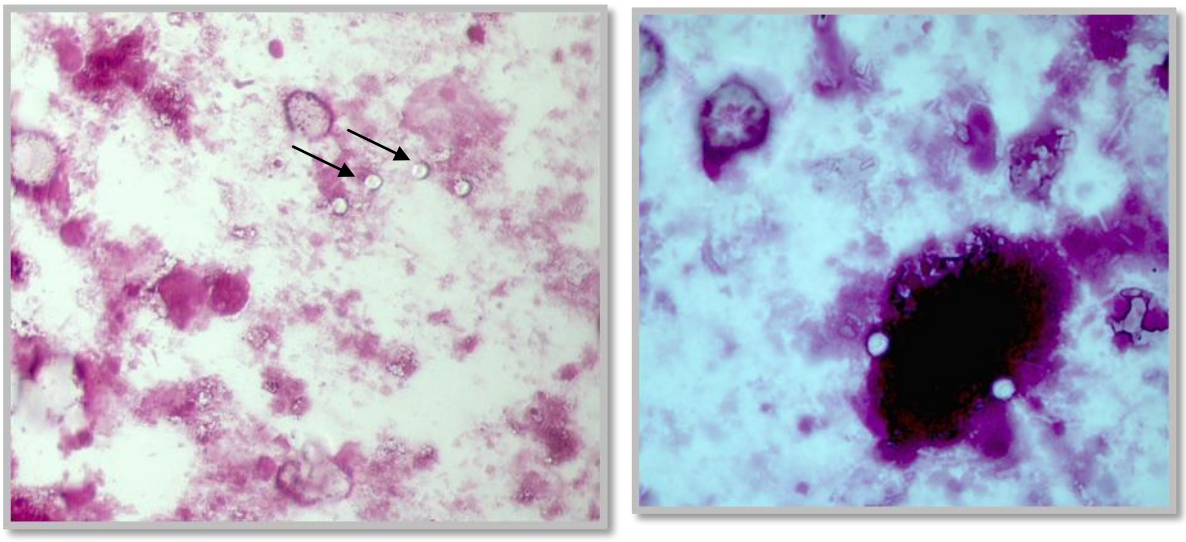
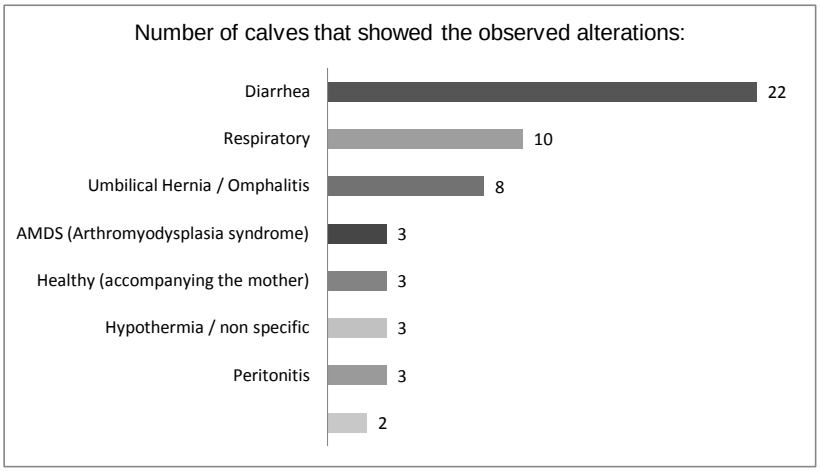


Fig. 14 and 15: Appearance of Cryptosporidium oocysts under the microscope after carbolfuchsin staining. In the positive slides, round, colourless, bright structures representing the oocysts are seen. Magnification: 400 x (left) and 600 x (right) (Original pictures).

Results:



Graph 2: Clinical signs or diseases shown by the calves. Note: some of the calves showed more than one of the alterations of the health status.

Total number of examined calves	**35**
Calves that showed signs of diarrhea	22 (100%)
Calves positive to *Cryptosporidium* spp.	8 (36%)
Calves that had diarrhea but due to a different source	14 (64%)

Table 1: Overview of the examined calves, the calves that showed diarrhea and the ones positive to *Cryptosporidium* spp..

Note: From the 22 calves with diarrhea, 8 were diagnosed with cryptosporidiosis. With other words, 36 % of the calves that showed diarrhea excreted *Cryptosporidium* spp oocysts.

Patient examination:

(For a more detailed view of the physical examination, please consult the tables 5 to 13 in the attachment; normal clinical examination parameters can be seen in the attached table no. 1 and hydration status was evaluated following the attached table no. 3 (2-4% slight dehydration; 6-8% moderate dehydration; >10% severe dehydration)).

Reference laboratory values can be seen on page 63.

Patient 1: Female Holstein calf. 2-3 days old.

- Patient number 1 was admitted at the clinic with watery diarrhea and slight dehydration. A slight alkalosis (pH = 7.405; base excess = 2.91), hyponatremia ($[Na^+]$ = 127 mmol/l) and a mild increase in lactate (1.7 mmol/l) was detected on laboratory analysis. Weight: 35 kg.
- **Treatment:** In the first day, an ear vein catheter was applied and renewed every 3 days. A continuous drip-like infusion of physiologic NaCl (0.9%) was installed and maintained during 9 days (from day 1 to day 9).

  Iron (Belafer ®), Vitamin A, D and E complex, Vitamin B complex, Vitamin E and selenium injections were given.
- On day 2 and 3, fecal consistency got normal (mushy to pasty), hydration status and mucous membranes reattained normal appearance (fast skin fold resolution and rosy mucous membranes). Intravenous fluid therapy was maintained.

  On days 4, 5 and 6, diarrhea relapsed.

  Fecal sample analysis diagnosed a positive excretion of *Cryptosporidium* oocysts +++
- **Treatment:** on day 5, the administration of halofuginone lactate (Halocur®) was started and maintained during 7 days (Day 5 to 11). Glucose 5 % was infused intravenously.
- On day 6 a new blood analysis showed a slight acidosis: pH 7.314; BE = -2.8 and a slight anemia: [Hemoglobin] = 4.6 mmol/l.

  Calculations: Bicarbonate requirements (mmol) = ΔBE (mmol/L) x Body Weight (kg) x 0.6 ⇔ HCO_3^- = 2.8 x 35 x 0.6 ⇔ HCO_3^- = 58.8 mmol

  Treatment: Bicarbonate (1 Bi-Pill®) per os
- On day 7, diarrhea symptoms decreased; lungs showed increased sounds and umbilicus was swollen and painful.
- On days 7, 8 and 9, treatment consisted of bicarbonate (1 Bi-Pill®) *per os* and subcutaneous enrofloxacin (Ursofloxacin® 2,5%).
- On day 10, diarrhea still existed but no increase in the lung sounds was detected and the umbilicus was not swollen nor painful. **Treatment:** bicarbonate (1 Bi-Pill®) *per os*.
- On days 11 and 12, the calf evidenced a good health status (except for a small umbilical hernia) and no further treatment (besides fluid therapy) was administered.
- The calf left the clinic on day 12.

Patient 2: Female, Holstein calf. 5 – 6 days old. Mothers were vaccinated against *Rotavirus*. Many calves at the same farm died already due to diarrhea. *Rotavirus* and *Coronavirus* were already detected on the farm. Pre-treatment: florfenicol (Resflor®) and cefquinome (Cobactan®).

- On day 1, the calf appeared with a good general health condition, showing only signs of diarrhea.
- Laboratory serum and blood analysis showed a slight alkalosis: BE = 5.2 mmol/l, a slight hyponatremia: 132 mmol/l, a slight hyperkalemia: 5.1 mmol/l and an increase in Lactate: 1.7 mmol/l.

  Blood analysis showed leukocytosis, relative neutrophilia, lymphopenia and monocytosis: Leukocytes 12 g/l; Neutrophyls 70 %; Lymphocytes 14 %; Monocytes 15.4 %.
- **Treatment:** A venous catheter was placed in the jugular vein and a drip-like physiological NaCl (0.9%) infusion connected. In addition, the calf received injections of iron (Belafer®), vitamin A, D and E complex, vitamin B complex and vitamin E and selenium.
- On day 2, the calf showed no signs of diarrhea but a slight increase in lung sounds was detected.
- Fecal sample results showed an infection with *Cryptosporidium* (+++) and *Coronavirus* (+).
- **Treatment:** halofuginone lactate (Halocur®) *per os* was started and given until day 8.
- From day 3 to 6, diarrhea relapsed and lung sounds got moderately increased, coughing and purulent nasal discharge was detected sporadically.
- **Treatment:** bromhexine (Bisolvon®) was administered in addition to halofuginone lactate (Halocur®) from day 3 to day 8.

  Antibiotherapy with cefquinome (Cobactan®) 2.5 % IM started on day 6 and continued during 5 days.
- On day 7, diarrhea stopped and the lung sound increase got less marked gradually until day 16.
- **Treatment:** Inhalation with saline solution was performed on days 12 and 13.
- The calf left the clinic on day 16.

Patient 3: Female, Simmental, 6 days of age. No mother vaccination. Pretreated with: selenium, N-butylscopolammonium bromide (Buscopan®), bicarbonate (Bi-Pill®) and meloxicam (Metacam®).

- The calf entered the clinic with watery diarrhea, severe dehydration and highly increased lung sounds coupled with abdominal breathing. Weight: 40 kg.
- Laboratory serum and acid-base data showed a high degree of acidosis: pH 7.051; BE: -18.3 mmol/l; hypoglycemia: [Glucose] = 3.0 mmol/l

  Calculations: Bicarbonate requirements (mmol) = ΔBE (mmol/L) x Body Weight (kg) x 0.6 ⇔ HCO_3^- = 18.3 x 40 x 0.6 ⇔ HCO_3^- = 439.2 mmol

- The fecal sample analysis diagnosed the presence of *Cryptosporidium* oocysts (+).
- **Treatment:** Venous catheter placement in the ear vein and renewal every 3 days. Connection of an infusion of physiologic NaCl (0,9%) solution (dropwise), sodium bicarbonate 8,4% (250 ml) and glucose 5 % (1L). An injection of Iron (Belafer®) was administered intramuscularly.

  Antibiotherapy with cefquinome (Cobactan®) 4,5% IV was started and maintained for 5 days.
- From day 2 to 6 an improvement in the severity of diarrhea and dehydration was detected (diarrhea went from watery to thin mushy and hydration status returned to normal on day 6).
- **Treatment:** On days 4 and 5, bicarbonate (Bi-Pill®) was given *per os*. On day 6, proteolytic enzymes (NekroVeyxym®) were administered intramuscularly and the infusion and venous catheter removed.

  Antibiotherapy was continued on days 6 and 7 with cefquinome (Cobactan® 2,5%) IM.
- Starting on day 7, no diarrhea was present but lung sounds continued moderately increased.
- On day 9 the calf was sent home.

Patient 4: Female, Galloway. 6 days of age. It was an isolated case in the farm, suffering from diarrhea since 3 days. Pretreatment: sodium bicarbonate, dexamethasone (Voren®), N-butylscopolammonium bromide (Buscopan®) and unspecified antibiotics.

- On day 1, the animal entered the clinic with watery diarrhea and moderate dehydration. Weight: 27,3 kg.
- Laboratory serum and acid-base analysis showed a severe acidosis: pH 7.033; BE: -16.6 mmol/l; hyperkalemia: [K+] = 7.4 mmol/l and an increase in Lactate: [Lactate] = 5.2 mmol/l

  Blood values indicated a Polycitemia (Hg = 10.5 mmol/l; Erythrocytes = 11.9 T/L; Hematocrit = 56.0 l/l), relative Neutrophilia (50.4 %), Lymphopenia (21.9 %), Monocytosis (27.2 %) and Eosinopenia.

  Highly increased kidney values: Urea: 25.4 mmol/L; Creatinine: 238 µmol/L

  Calculations: Bicarbonate requirements (mmol) = ΔBE (mmol/L) x Body Weight (kg) x 0.6 ⇔ HCO_3^- = 16.6 x 27.3 x 0.6 ⇔ HCO_3^- = 271.9 mmol
- **Treatment:** A jugular vein catheter was placed (renewed every 3 days) and a continuous drip infusion with physiological saline was connected, together with sodium bicarbonate 8.4% IV (500 ml) and glucose 5% IV (1 L).

  Iron (Belafer®), Vitamin A, D, E complex, Vitamin B complex, Vitamin E and selenium were administered.

- Diarrhea continued until day 5 in the clinic, attaining mushy to pasty consistency on the following days. During this time, cefquinome (Cobactan®) 2,5% was administered intramuscularly.
- On day 2, Acidemia: pH = 7.389; BE = 9.0 mmol/l and hypernatremia: [Na+] = 155 mmol/L were detected. In addition to hypocalcemia: [Ca+] = 0.86 mmol/L
  Fecal analysis diagnosed Cryptosporidiosis (+).
- **Treatment:** calcium borogluconate (Kalzibosel®) SC and Sterofundin® ISO IV (dropwise) (500 ml) were administered.
  Antibiotherapy from day 2 to day 6 consisted of enrofloxacin (Ursofloxacin) 5% SC.
- On day 3, the kidney values were normal: Urea: 2.8 mmol/L; creatinine: 66 µmol/L
- The infusion of physiologic saline was maintained until the last day of the treatment (day 7).

After going home: The calf was readmitted the next day, due to relapse and worsening of the clinical signs.

- On the day of readmittance (day 8), watery diarrhea, slight dehydration and pale mucous membranes were present.
- Laboratory serum and blood analysis showed: a very slight acidosis (pH 7.322; BE: -1.7 mmol/L); a slight hyponatremia ($[Na^+]$ = 128 mmol/l); hyperkalemia ($[K^+]$ = 5.2 mmol/l).
  Fecal analysis: *Cryptosporidium* ++
  Calculations: Bicarbonate requirements (mmol) = ΔBE (mmol/L) x Body Weight (kg) x 0.6 ⇔ HCO_3^- = 1.7 x 27.3 x 0.6 ⇔ HCO_3^- = 27.8 mmol
- **Treatment:** Iron (Belafer); Vitamin A, D, E complex; Vitamin B-complex; bicarbonate (1 Bi-pill®) per os.
- On day 9 and 10 the diarrhea, dehydration and pale mucous membranes continued, and, in addition, increased lung sounds were diagnosed.
- **Treatment** on day 9 consisted of bicarbonate (1 Bi-pill®) PO and oral rehydration fluids.
  From day 9 to 19, Diarsanyl® was administered per os.
  And on day 10, a venous catheter was placed in the jugular vein and a drip infusion of physiologic saline (0.9%) started. Bicarbonate (1 Bi-pill®) was given *per os* on day 10 and 11.
  Halofuginone lactate (Halocur®) PO was started and maintained until day 16.
- Starting from day 11, diarrhea stopped and the signs between day 11 and 13 were mainly of respiratory nature (slightly increased respiratory sounds).
- On day 12 the venous catheter was removed and intravenous fluid therapy was stopped.

- Slightly increased lung sounds continued until day 17.
- **Treatment** on day 14 consisted of *Echinacea* per os; Vitamin B complex; Vitamin A, D, E complex. And on day 15, 16 and 17 of bicarconate (1 Bi-Pill®) PO
- On day 16 and 17 the umbilicus showed some degree of pain. Following this, the ultrasound of the umbilicus showed following results:

  Distally, on the skin area, some hyperechogenic content was present. Liver echography showed no alterations. Hepatic vein could not be followed cranially.
- From day 17 to 19, **treatment** with Echinacea® *per os* and a topical cooling gel (Compagel®) on the neck were performed.
- On day 19, the calf went home.

Patient 5: Red-Holstein, male. 14 days of age. Diarrhea since 7 days. Pretreatment: halofuginone lactate (Halocur®), vitamin E and selenium, N-butylscopolammonium bromide (Buscopan®), loperamide (Diacure®) and vitamin B12 (Catosal®).

- On day 1 the calf entered the clinic with watery diarrhea, slight dehydration and slightly increased lung sounds. Weight: 45 kg.
- Laboratory serum and acid-base results showed a very slight acidosis: BE -1.3; Increased lactate: [Lactate] = 2.3 mmol/l

  Fecal analysis diagnosed: *Cryptosporidium* +++ and *Rotavirus*: positive

  Calculations: Bicarbonate requirements (mmol) = ΔBE (mmol/L) x Body Weight (kg) x 0.6 ⇔ HCO_3^- = 1.3 x 45 x 0.6 ⇔ HCO_3^- = 35.1 mmol
- **Treatment:** A venous catheter was placed in the jugular vein and a physiologic saline (0.9%) solution (drop wise) was connected.

  Injections of iron (Belafer®); vitamin B complex; vitamin A, D, E complex;

  Cefquinome (Cobactan®) 2,5% intramuscularly and bicarbonate (1 Bi-Pill®) *per os* were administered from day 1 to 5.
- Watery diarrhea continued until day 4, stopping on day 5, when the calf was sent home.

Patient 6: Red-Holstein, male. 7 days of age. Diarrhea since several days. No pre-treatment was performed at the farm.

- On day 1, thin mushy diarrhea, slight dehydration and mild fever (39.8ºC) was detected. Weight: 40 kg.
- Laboratory serum and blood analysis showed a slight acidosis: pH = 7.304; BE: -2.3. And fecal analysis: *Cryptosporidium* +++

  Calculations: Bicarbonate requirements (mmol) = ΔBE (mmol/L) x Body Weight (kg) x 0.6 ⇔ HCO_3^- = 2.3 x 40 x 0.6 ⇔ HCO_3^- = 55.2 mmol

- **Treatment:** A jugular vein catheter was introduced and a continuous drip infusion of physiologic saline (0.9%) was connected. Injections of iron (Belafer®); vitamin A, D, E complex and vitamin B complex were given.
  On day 1 and 2, respectively, 1 Bi-Pill® (bicarbonate) was administered *per os*.
  Antibiotherapy from day 1 to 5 consisted of cefquinome (Cobactan®) 2.5% intramuscularly.
- Already on day 2, the general health status seemed normal only on day 4, an episode of fever (39.7ºC) occurred. The calf left the clinic on day 5.

Patient 7: Holstein black and white, female. 8 days of age. Sporadic case at the farm, pre-treated with meloxicam (Metacam®) and marbofloxacin (Marbocyl®).

- On the first day, the calf showed thin mushy to watery diarrhea, slight dehydration, pale mucous membranes and mild hypothermia (37.9ºC). The lung sounds were slightly increased. Weight: 40 kg.
- Laboratory serum and blood analysis showed a moderate acidosis (pH 7.278; BE: -6.0); slight hyponatremia ($[Na^+]$ = 126 mmol/l). Blood analysis evidenced a slight anemia (Hemoglobin = 4.8 mmol/l). And fecal analysis: *Cryptosporidium* +++
  Calculations: Bicarbonate requirements (mmol) = ΔBE (mmol/L) x Body Weight (kg) x 0.6 ⇔ HCO_3^- = 6.0 x 40 x 0.6 ⇔ HCO_3^- = 144 mmol
- **Treatment:** A jugular vein catheter was introduced and a continuous drip infusion of physiologic saline (0.9%) was connected.
  Sodium bicarbonate infusion 8.4% IV (250 ml) was performed and iron (Belafer®), vitamin A, D, E complex, vitamin B complex, vitamin E and selenium were injected.
  From day 1 to 8, cefquinome (Cobactan®) 2,5% was administered intramuscularly.
- On day 2, diarrhea was still present, but stopped on day 3.
- The calf was sent home on day 12 with no altered health issues.

Patient no. 8: Black and white Holstein, male. 12 days of age. Pretreated with Iron, Vitamin complexes B, ADE and Selenium; meloxicam (Metacam®) and penicillin (Veracin comp®).

- On the first day, watery to thin mushy diarrhea, slight dehydration and slight hypothermia (37.8ºC) was present.
- Laboratory serum and acid-base analysis showed severe metabolic acidosis (pH 7.104; BE = -15.5 mmol/l); hyponatremia ($[Na^+]$ = 126 mmol/l); hyperkalemia ($[K^+]$ = 6.6 mmol/l)
  Fecal analysis: *Cryptosporidium* +
  Calculations: Bicarbonate requirements (mmol) = ΔBE (mmol/L) x Body Weight (kg) x 0.6 ⇔ HCO_3^- = 15.5 x 38 x 0.6 ⇔ HCO_3^- = 353.4 mmol

Treatment: An ear catheter was introduced (and exchanged every 3 days) and a continuous drip infusion of physiologic saline (0.9%) was connected. In addition, sodium bicarbonate 8.4% solution (500 ml), Sterofundin® ISO and glucose 5% (500 ml) were infused.

- On day 2, laboratory serum and blood analysis showed a very slight acidosis (pH 7.353; BE: -3 mmol/l).

  Calculations: Bicarbonate requirements (mmol) = ΔBE (mmol/L) x Body Weight (kg) x 0.6 ⇔ HCO_3^- = 3 x 38 x 0.6 ⇔ HCO_3^- = 68.4 mmol
- **Treatment:** 2 Bi-Pill® (bicarbonate) PO (1 in the morning and 1 in the evening); amoxillin and clavulanic acid (Synulox® RTU).
- Diarrhea and dehydration continued until day 3 and on day 3 a single episode of fever was detected.
- **Treatment:** Glucose 5% was infused and oral rehydration fluid administered from day 2 to 4.

  On day 3, 2 Bi-Pill® (1 in the morning and 1 in the evening) were given orally and Synulox® RTU was injected intramuscularly.

  Antibiotherapy with cefquinome (Cobactan®) 2,5% was administered intramuscularly from day 3 to 7.
- On days 6 and 7, tarsal joints were slightly painful. The calf was sent home.

Normal values (Kaske & Kunz, 2003)

pH 7,33 – 7,37
BE -2 – 2 mmol/l
$[Na^+]$: 135 – 145 mmol/l
$[K^+]$: 3,5 – 4,5 mmol/l
$[Ca^{2+}]$: 2,1 – 2,8 mmol/l*
[lactate] < 1,5 mmol/l
[Glucose]: 5,0 – 8,0 mmol/l
Urea: 2,0 – 5,5 mmol/l;
creatinine: < 150 mmol/l

Erythrocytes: 5,0 – 10,0 T/L
Hematocrit: 0.25 – 0.37 l/l
[Hemoglobin]: 4,96 – 7,45 mmol/l
Leukocytes: 5 – 10 g/L;
Neutrophyls: 25 – 48%;
Lymphocytes: 45 – 65 %; Monocytes: 2 – 6 %; Eosinophyls: 1-10 %

Table 2: Normal laboratory values for new-born calves.

* Merck Sharp & Dohme Corp., 2011.

Discussion:

After analyzing the obtained results it was discovered that the most frequent disorder shown by the examined calves was diarrhea (22 out of 35). This confirms the previously described importance of diarrhea in the rearing of calves (Foster & Smith, 2009)

A high proportion of the calves suffering from diarrhea was excreting *Cryptosporidium* oocysts (36%). This finding is also according to current beliefs, which state that cryptosporidiosis is one of the most important diarrhea causing agents in neonatal calves (de Graaf *et al.* 1999b; O'Handley & Olson, 2006).

In 2000, Krull determined the average infestation rate of calves with cryptosporidiosis in Germany. Results showed a rate of 20 to 30%. Siebert and Gründer also did a study, which showed a prevalence of cryptosporidiosis cases in diarrheic calves of nearly 40% (Iben, 2004). However, these results are not comparable to the present study due to different trial conditions (clinic and on field).

Affected animals ranged in age from 2-3 days to 14 days, confirming previous statements which say that mostly calves with 5 to 14 days of age are affected (Rommel *et al.* 2000; Kaske & Kunz, 2003; Olson *et al.* 2004; Thompson *et al.* 2008; O'Handley & Olson, 2009).

It is, however, important to focus that only calves suffering from diarrhea were tested for the presence of *Cryptosporidium* oocysts. Other calves without signs of diarrhea could be possibly infected with *Cryptosporidium* without showing any clinical signs (assympthomatic carriers) (de Graaf *et al.* 1999b; Naciri *et al.* 1999; Tzipori & Ward, 2002).

After carbolfuchsin staining the smears were observed immediately. This way the oocysts were evidenced as bright, white, refractile and round formations in front of a pink background. The stain acts as a negative stain in this case and, even though it is of easier execution, it is much less reliable than the modified Ziehl-Neelsen stain for instance (Fayer *et al.* 2000).

However, as the technique is of a much faster execution, the number of examined samples is higher (when comparing to the modified Ziehl-Neelsen technique) and the process is much cheaper due to the use of less staining material, it was considered advantageous.

After the determination of the positivity to cryptosporidiosis, those calves were examined in a more detailed way, considering the evolution of the disease and its outcome.

Routine treatment of the 8 calves upon arrival included a venous catheter and a continuous drip infusion of physiologic saline and iron and vitamins (B, A, D and E) injections, to rehydrate and balance the sodium and chloride concentration in the blood and to increase the overall vitality of the calf, respectively.

Selenium was also administered as a routine treatment. This mineral is considered to be an essential antioxidant in combination with vitamin E that protects cells against the oxidizing effect exerted by the metabolic products. Its deficiency in calves gives rise to several

degrees of myopathy, demonstrated by weakness, difficulties in suckling and standing and even respiratory distress or cardiac rhythm alteration that may lead to collapse and death (Duchy College *et al.* 2006). It is highlighted at this point, as selenium deficiency is frequent in the german region of Hessen in which this clinical case study took place, leading to cases of calf weakness, lower feed intake and worsening of the clinical condition.

In Patient 1, as the calf started to show diarrhea symptoms only a few days before, was not dehydrated and still consumed milk relacer, showing a good general health status, Halocur® (halofuginone lactate) was administered during 7 days.

Halocur®, a yellow liquid containing 0.5 mg/ml halofuginone lactate in an aqueous suspension (MSD Animal Health. Intervet International B. V., 2009), is currently used because of its possible function in the reduction of the signs of diarrhea and the amount of excreted oocysts (Villacorta *et al.* 1991; Kaske & Kunz, 2003; Olson *et al.* 2004; Jarvie *et al.* 2005; O'Handley & Olson, 2006); even though it did not show consistently positive results (Naciri, Mancassola, Yvoré & Peeters, 1992; Peeters, Villacorta, Naciri & Vanopdenbosch, 1993).

Due to a slight acidosis in the middle of the treatment period (day 6) (requiring 58.8 mmol of bicarbonate), oral sodium bicarbonate in form of a tablet (Bi-Pill ®) was given to the calf in the following 5 days. This compound is used to correct the acid-base status and also to increase the voluntary milk intake (Fuettern und fit – Das Beste direkt vom Tierarzt, 2011). Intravenous sodium bicarbonate was not considered to be necessary, as the acidosis was not very pronounced.

The administered oral bicarbonate was meant to balance the existing acidosis, as well as to prevent the development of a new acidotic status due to the persistence of diarrhea.

On days 7 to 9, Ursofloxacin® was administered. Containing enrofloxacin, an antibiotic compound belonging to the group of fluorquinolones which acts by inhibition of bacterial DNA gyrase, it has a broad spectrum of action against gram positive and gram negative bacteria. Side effects are not very frequent, however, in growing animals it can affect the cartilages and in dehydrated animals it can show nephropathic activity. Its use in neonatal diarrheic calves should, hence, be restricted to calves without or only slight signs of dehydration (Plumb, 2008; Mar Vista Medical Center, 2009; Infectious Disease Epidemiology Section, Office of Public Health, Louisiana Department of Health and Hospitals). Here, its use was directed to the prevention of a possible septicemia, but also to stop the emerging respiratory disease and omphalitis.

On day 12 the calf was sent home with a good general health condition, without diarrhea, dehydration or respiratory disease. The animal recovered well after oral and intravenous fluid therapy, oral sodium bicarbonate, Halocur® and broad spectrum antibiotic therapy.

Patient number 2 arrived at the clinic after being pretreated with Resflor® and Cobactan® 2,5% at the farm.

Resflor® is an antimicrobial compound containing florfenicol and being a very fast and effective treatment against BRD (Bovine respiratory disease). As it acts mainly against respiratory pathogens (*Mannheimia haemolytica, Pasteurella multocida, Histophilus somni* and *Mycoplasma bovis*) it seems to have no significance in cases of diarrhea and was probably applied due to a concurrent respiratory infection (Apifarma, 2007; Van Donkersgoed, Berg & Hendrick, 2011). Its use under 6 weeks of age and in cases of dehydration is not indicated, as it is in this case (Apifarma, 2007). Its use in this case, was not considered a correct decision.

Cobactan® 2,5% is a product containing cefquinome. This 4th generation cephalosporin is known for its broad spectrum efficacy in respiratory disorders, foot infections, mastitis and, most important in this case, *E. coli* septicemia in calves (Apifarma, 2007; MSD Animal health. Intervet International B.V., 2009). Calves should be treated during, at least, 5 days (Apifarma, 2007).

As in the farm already many calves were affected by diarrhea and the presence of *Rotavirus* and *Coronavirus* was previously diagnosed at the farm, one of these viruses may have been present and at least in part responsible for the diarrhea signs.

The calf entered in a good general health status showing only diarrhea. In addition, there was a slight alkalosis, hyponatremia, hyperkalemia, an increase in lactate and leucocytosis.

After the routine fluid therapy and injections, diarrhea improved but relapsed later on. Fecal analysis identified a mixed infection of *Cryptosporidium* (+++) and *Coronavirus.*

A treatment with Halocur® was started on day 2 and maintained for 7 days, as the hydration status was normal and the calf's vitality was good.

From day 3 to day 8, Bisolvon® was administered. This bronchosecretolyticum is applied to liquefy the bronchial mucosal secretions in order to help the expectoration and improve the emerging respiratory signs (Tierarzneimittel Kompendium der Schweiz. Clinipharm Clinitox, 2006).

From day 6 to 10, antibiotherapy with Cobactan® 2,5% was started in order to avoid the appearance of septicemia and stop the development of a respiratory disorder.

Saline inhalation to help the discharge of fluids and expectoration was performed on days 12 and 13. Following this treatment, mucous discharge was seen on the following days, evidencing a possible positive result.

On day 16, finally, the calf was released from the clinic without diarrhea, a good general condition and only mild respiratory signs.

In this case, the calf improved after fluid therapy, Halocur®, Cobactan® 2,5% and Bisolvon® administration as well as saline inhalation. As there was no metabolic acidosis, in this case no alkalinizing sodium bicarbonate had to be administered.

Patient number 3 entered the clinic after 3 days of weakness and after being treated at the farm with selenium, Buscopan®, Bi-Pill® (sodium bicarbonate tablet) and Metacam®.

Buscopan®, containg the active substance N-butylscopolammonium bromide, is currently used as a spasmolytic and anticholinergic drug to stop spasms in the digestive system primarily in horses but also applicable in the bovine species. Especially in different cases of colic that might be related to diarrhea (Tierarzneimittel Kompendium der Schweiz. Clinipharm Clinitox, 2003; Drug Information Online. Drugs.com, 2011).

Metacam® is a non steroid anti-inflammatory drug containing meloxicam. It is used as the only currently licensed NSAID in cases of calf diarrhea together with fluid therapy and has good effects in the improvement of the calf's health condition by increasing feed and fluid intake and the overall intestinal function (Apifarma, 2007; Boehringer Ingelheim Vetmedica GmbH, 2011). In a study performed by Philipp, Schmidt, Düring and Salomon (2003) improvement of the rectal temperature, general condition, behavior, feed intake, dehydration, pain signs and fecal consistency was detected.

In severely dehydrated calves it should be used with caution as is has a nephrotoxic potential (Apifarma, 2007). In this study, even though the use of Metacam® is considered to be beneficial in the improvement of the calves' health, it was not always used due to the risk in cases of dehydration and to its high price.

The patient was very weak and showed diarrhea, respiratory signs, a high degree of dehydration and a slight anemia. On laboratory analysis, a severe acidosis (BE= -18,3 mmol/l) was detected (with a possible life threatening potential) and hypoglycemia. Fecal analysis showed a small number of *Cryptosporidium* oocysts under the microscope (+).

After calculating the required bicarbonate amount to balance the acidosis (439,2 mmol), in addition to the routine saline drip-infusion, 250 ml of a 8,4% sodium bicarbonate infusion (which would cover 250 mmol of the required amount) was administered. No more concentrated solution was administered, as the animal was showing severe respiratory signs which would impair the exhaling of the carbon dioxide formed in the reactions (Koch, 2004; Naylor *et al.* 2006).

Glucose was administered in order to correct the detected hypoglycemia; and the slight anemia was approached with an iron injection.

From day 1 to 5, intravenous Cobactan® 4,5%, containing the broad spectrum 4th generation cephalosporin cefquinom, was administered intravenously to avoid the appearance of colisepticemia (Tierarzneimittel Kompendium der Schweiz. Clinipharm Clinitox, 2008). This compound is, comparing to the routinely used intramuscular 2,5% solution, much faster and more potent in its action, having the disadvantage of being more expensive.

As the diarrhea continued and in order to avoid the reappearance of metabolic acidosis, oral sodium bicarbonate in tablet form (Bi-Pill®) was given on days 4 and 5.

Until day 6 of treatment, hydration assumed a normal degree and diarrhea got less fluid due to the effect of the fluid therapy and possible resolution of infection. Following this, the venous infusion of physiologic saline was stopped and the catheter removed.

As, even though, the risk for septicemia persisted, antibiotherapy was continued on day 6 and 7 with intramuscular cefquinome (Cobactan®) 2,5%.

On day 6, NekroVeyxym® was injected. This compound consists of proteolytic enzymes which act on the immune system. It is known to stimulate immunity, cause fibrinolysis, cause the breakdown of inflammatory products and necrosis, inhibit the growth of pathogenic organisms, support antibiotherapy, and decrease fever (Veyx Pharma GmbH, 2007).

Finally, on day 9, the calf was sent home with a good general health status and only slightly increased respiratory sounds.

The improvement in this case was due to the action of rehydration, sodium bicarbonate, glucose, Cobactan® 4,5% and Cobactan® 2,5% and Nekroveyxym® (possibly together with the previously administered Buscopan®, selenium and Metacam®).

Patient number 4 was an isolated case in the farm, suffering from diarrhea since 3 days. At the farm, the calf was pretreated by the farmer with sodium bicarbonate, Voren®, Buscopan® and unspecified antibiotics.

Voren® contains dexamethasone–21-isonicotinate as its active ingredient. It is a potent, long-acting corticosteroid with glucogenic and anti-inflammatory functions (Böhringer-Ingelheim, 2005; Apifarma, 2007). As Lopez *et al*. (1975) showed, corticosteroids should not be used in cases of calf diarrhea, as they suppress the immune system and also due to the fact that their concentration is already increased in diarrheic calves. The use of Voren® in this case was, hence, not a good decision from the farmer.

At the day of entrance, the calf was presented with watery diarrhea and a moderate degree of dehydration. In addition, a severe acidosis (BE = -16.6 mmol/l) with a high increase in lactate (5.2 mmol/l) and a polycitemia was diagnosed. Relative neutrophilia, lymphopenia, monocytosis and eosinopenia show the marked inflammatory response and infiltrations.

Due to dehydration, kidney perfusion got compromised and accumulation of urea and creatinine was detected in a high degree.

Fluid therapy with physiologic saline as a continuous drip infusion and injection of iron, selenium and vitamins was done as the routine first approach.

The required bicarbonate of 272 mmol was balanced with the administration of concentrated 8.4% sodium bicarbonate solution. Glucose was administered as an approach to weakness due to hypoglycemia.

Also Cobactan® 2.5% (cefquinome) was administered during 5 days in order to avoid septicemia.

On the second day of treatment, the calf showed metabolic alkalosis (BE = 9.0 mmol/l) showing a possibly excessive IV treatment with sodium bicarbonate on the first day in the clinic. In addition, on the second day hypernatremia (possibly due to restricted ingestion of water), hypocalcemia (related to diarrhea losses) and a slightly positive cryptosporidiosis finding (+) was detected.

Kalzibosel® (calcium borogluconate) was injected subcutaneously to increase the serum calcium concentration, in order to avoid muscular weakness and maintain the suckling force.
Sterofundin® ISO, a very complete mineral solution containing sodium, chloride, potassium and magnesium as well as acetate, was infused as a potent rehydrating solution with slight acid-base correction ability, to allow the balancing of the homeostasis (Braun Melsungen, 2008)
In addition to the Cobactan® (cefquinome) administration started on day 1, an additional antibiotic (Ursofloxacin® 5% - enrofloxacine) was administered to increase the efficacy spectrum and fight secondary infections and septicemia.
On day 3, the fluid therapy helped improving kidney perfusion and kidney values (urea and creatinine) attained physiological values. Fluid therapy was, however, maintained until the end of the stay in the clinic to warrant exclusion of relapses.
The calf improved, diarrhea disappeared and on day 7 the general condition of the calf was so good that it could be sent home.
No Halocur® (halofuginone lactate) was used in this case as in the beginning of treatment the calf showed marked dehydration, weakness (not able to drink by itself) and affected kidney function.
After going home, however, the calf was readmitted at the clinic. Watery diarrhea relapsed together with slight dehydration. Laboratory data showed a very slight acidosis (BE= -1.7 mmol/l), hyponatremia and hyperkalemia. A fecal analysis was performed again and *Cryptosporidium* oocysts detected in a higher amount than at the first admittance (++). Possibly the infection had just started on the first stay at the clinic and multiplication took place in the meanwhile.
The routine injections followed together with oral sodium bicarbonate, as acidosis was only slight (required amount of bicarbonate was only 27.8 mmol). Bi-Pill® was administered per os during 4 days (day 8 to 11) to balance new developing acidosis due to the persistent diarrhea.
As the degree of dehydration was mild, rehydration was performed only orally through balanced oral electrolyte solutions.
Diarsanyl® was administered and maintained for 11 days. It is known as an antidiarrheicum that improves the resorptive capacity of the gut especially during or after the occurrence of diarrhea (Apifarma, 2007). In this case it was used as diarrhea was improving but dehydration was still present.
As the dehydration status did not improve through oral fluid therapy, a venous catheter was placed, a saline continuous drip infusion connected and the infusion started.
On day 10, as dehydration was still only slight and the health status and vitality of the calf was good, a treatment with Halocur® was started and maintained for 7 days.

On day 12, when hydration status reattained a normal degree, IV fluid administration was stopped and the venous catheter was removed. Further rehydration could be attained orally as the calf was vital and able to suckle.

Mucous membranes appeared slightly reddened on day 14 indicating a possible start of a septic condition. Echinacea®, vitamin B complex and vitamins A, D, E were given to increase natural resistance and vitality.

Echinacea®, a natural compound obtained from the *Echinacea* plant, and with immune-stimulating properties, is used in upper respiratory tract conditions or other kind of inflammatory processes and is very well tolerated (Marsden S. *et al.* 2007)

On day 16, also the umbilicus showed pathologic alterations. Ultrasound examination, however, did not evidence any structural abnormality, which suggests no major pathological condition.

Finally, on day 19, the calf recovered completely and was sent home.

The calf took longer to improve and only after 2 visits it attained normal health status. Oral and intravenous fluid therapy, sodium bicarbonate, iron, selenium, calcium and vitamins, antibiotics (cefquinome and enrofloxacin) and Halocur® were necessary to attain this improvement. Possibly, several factors weakened it in a higher degree so that it could not fight the infection as well as the other calves.

The calf was much smaller in size and of a beef-breed kept outside. In addition, this study was performed in winter and the calf would be more prone to hypothermia and consequent weakening.All these points could have weakened the calf to a higher extend when comparing to the others.

Beef calves, although being less frequently affected by the disease, are more likely to develop severe cases of cryptosporidiosis when compared to dairy calves, resulting in up to 30% mortality. An explanation could be the higher incidence of selenium deficiency in beef calves when compared to dairy calves (which weakens immunity status) (Olson *et al.* 2004; O'Handley & Olson, 2006). However, as serum selenium was normal in this particular calf, it is unlikely to be the reason. In addition, herd immunity is frequent in dairy but not in beef cattle, so lower herd immunity might have some relevance (Bopp, 2003).

Patient number 5 was admitted at the clinic after showing diarrhea for 7 days and being pre-treated by the farmer with Halocur® (halofuginone lactate), vitamin E and selenium, Buscopan®, Diacure® and Catosal®.

Diacure® is a compound containing loperamide, an antidiarrheic drug which acts by decreasing the peristaltic motility and increasing the absorptive activity of the intestine (Drugs.com. Drug Information Online, 2011).The use of any drugs that diminish the intestinal motility is not recommended as they increase the time the agent is in contact with the mucosa and may worsen the lesions (Kaske & Kunz, 2003; Constable, 2009). The decision of the farmer was not, hence, entirely correct.

Catosal® is a metabolism regulator containing vitamin B12 and phosphoric acid. In calves it is used in cases of nutrition deficiencies and muscular weakness and improves greatly the vitality of the affected animals (Bayer Healthcare- Saúde Animal, 1990; Apifarma, 2007).

On the day of entrance, the calf showed watery diarrhea, slight dehydration and slightly increased lung sounds. In addition, laboratory data showed a very slight acidosis (BE = -1,3 mmol/l) and a moderate increase in lactate (2,3 mmol/l). Fecal analysis showed an intense infection with *Cryptosporidium* (+++) together with an infection by *Rotavirus.*

Routine infusion and injections were performed.

In order to fight the emerging respiratory disease and to avoid the appearance of septicemia, Cobactan® 2,5% was administered during 5 days.

Acidosis, as it was only mild (requirement of 35.1 mmol bicarbonate), was treated orally by the administration of sodium bicarbonate in the form of a tablet (Bi-Pill®).

On day 5 the calf was sent home showing no signs of diarrhea and being only slightly dehydrated, with the indication of further oral rehydration at the farm.

As the calf was already pretreated with halofuginone lactate, its administration was not performed at the clinic; also because it is more indicated as a preventive measure or in the first days of diarrhea and not after 7 days of diarrhea and a dehydrated status, to avoid undesired side effects (Kaske *et al.* 2003; MSD Animal Health. Intervet International B. V., 2009).

The calf improved after fluid therapy, iron, selenium and vitamin administration, Cobactan® 2,5% and oral sodium bicarbonate.

When patient number 6 entered the clinic, its health status was moderately altered showing moderately severe diarrhea, slight dehydration and mild fever. In addition, a slight acidosis (BE = -2,3 mmol/l) and the presence of *Cryptosporidium* oocysts in a fecal sample (+++) were detected.

After the routine establishment of the continuous infusion with physiologic saline and the injection of iron, selenium and vitamins, acidosis was controled by the oral administration of sodium bicarbonate (Bi-Pill®) (low bicarbonate requirements: 55.2 mmol) during 2 days. An attempt to prevent septicemia was done by the administration of Cobactan® 2.5% (cefquinome).

Already on the second day of treatment, the calf improved its health status as the feces attained normal consistency. The swollen articulation on the second day and the episode of fever on day 4 were not considered of importance as they improved on the next days.

Halofuginone lactate was not administered in this patient as the diarrhea existed already since several days and, due to the dehydration, a potential nephrotoxic effect should be considered (Kaske & Kunz, 2003; MSD Animal Health. Intervet International B. V., 2009).

Improvement occurred after fluid therapy, iron, selenium and vitamin administration, oral sodium bicarbonate and Cobactan® 2,5% injection.

Patient number 7 was a sporadic case at the farm and arrived after being pre-treated with Metacam® and Marbocyl® for anti-inflammatory and antibiotic treatment.

Marbocyl® contains marbofloxacin and is a broad spectrum antibiotic with a similar action to enrofloxacin (Ursofloxacin®), but mainly directed to respiratory disorders (Apifarma, 2007; Tierarzneimittel Kompendium der Schweiz. Clinipharm Clinitox , 2008).

At the clinic, the calf showed diarrhea (thin mushy to watery), slight dehydration and hypothermia, slightly increased respiratory sounds and pale mucous membranes. A moderate acidosis (BE = -6 mmol/l), slight hyponatremia and slight anemia were detected. Fecal analysis evidenced *Cryptosporidium* oocysts (+++).

In addition to the routine saline infusion and the injection of iron, selenium and vitamins, intravenous infusion of sodium bicarbonate was attempted to correct metabolic acidosis.

In this patient, intravenous sodium bicarbonate was administered as the requirements were of moderate degree (144 mmol). Hence, 250 ml of a 8,4% solution were administered, taking into account further losses due to persistant diarrhea.

Cobactan® 2,5% (cefquinome) was administered during 8 days to avoid septicemia and also to stop the emerging respiratory disease.

On day 3, the calf attained a good health status and did not show any signs of diarrhea. It was only sent home on day 12 to assure its recovery.

Improvement was, hence, observed after fluid therapy, iron, selenium and vitamins, sodium bicarbonate and Cobactan® 2,5% (probably supported by the Metacam® and the Marbocyl® from the pre-treatment).

Finally, patient number 8 was admitted at the clinic with diarrhea, slight dehydration and hypothermia. Severe acidosis (BE = - 15,5 mmol/l), hyponatremia and hyperkalemia constituted the laboratory findings. Cryptosporidium was detected in the fecal sample with only a slight positivity +.

The farmer had already pretreated the animal with iron and vitamin injections, Metacam® (anti-inflammatory and pain control) and Veracin compositum®.

Veracin compositum® is a long life penicillin with spectrum mainly on gram positive β-lactamase sentitive bacteria (Albrecht GmbH, 2006). As cases of diarrhea should be medicated with antibiotics directed to the prevention of septicemia and the mainly involved bacteria is *E. coli*, the administration of this antibiotic can not be considered to be ideal. Preferably an antibiotic against gram negative bacteria should be used (Constable, 2004).

Routine treatment on the first day with infusions and injections was performed.

In order to correct the severe acidosis (353,4 mmol of bicarbonate requirements), a high amount (500 ml) of sodium bicarbonate (8,4% solution) was infused at a very low rate, as fast infusions should be avoided in dehydrated and acidotic calves. They can cause hyperosmolality of the extracellular fluid, hypernatremia, hypocalcemia, and paradoxic

intracellular and CSF acidosis, and being especially harmful in cases of concurrent respiratory disease (Koch, 2004; Naylor *et al.* 2006).

Following the results obtained by Blume (2007), though, no marked difference was seen between the fast administration of concentrated sodium bicarbonate and the administration of sodium bicarbonate diluted in isotonic sodium chloride. This author states that a bolus of a 8.4% solution of sodium bicarbonate can be given at a fast rate without causing any complications.

Glucose 5% was infused to supply energy in order to fight hypothermia and general weakness. Sterofundin ISO®, an isotonic rehydration solution containing sodium, chloride, potassium, other electrolytes and acetate, was administered to help the rehydration and alkalinization of the calf.

On the second day of treatment, acidosis assumed a slight degree (bicarbonate requirements lowered to 68,4 mmol) and correction of the acid-base status could be attempted through the oral way (by administration of Bi-Pill® tablets on day 2 and 3).

A treatment with Synulox RTU® was attempted (a compound containing amoxicillin and clavulanic acid with a wide spectrum of gram negative and gram positive bacteria) to avoid septicemia (Pfizer Laboratories Ltd., 2004; Apifarma 2007). However, as the clinical signs did not improve and the general health status even worsened, antibiotherapy was switched to Cobactan® 2,5% and administered during 5 days from day 3 to day 7 of the treatment. As the condition improved after the antibiotic change, Cobactan® might be a better choice in the treatment of calf diarrhea complications.

On days 6 and 7, pain in the tarsal joints was detected which shows a possible involvement of the articulations (as a sign of septicemia). However, as the pain was not pronounced, the calf had no fever and the general health situation was good, the patient was sent home with the indication that the farmer should contact the clinic in case of worsening of the clinical signs (inability to stand, weakness).

The patient improved with the fluid therapy, sodium bicarbonate, glucose, sterofundin® ISO, Synulox RTU® and Cobactan® 2,5%.

Cross-relating the findings of all 8 patients, it could be observed that all calves improved and could be sent home after a period of time varying from 5 to 19 days. These findings show the efficacy of the treatment and the self-limiting character of the infection (Kaufmann *et al.* 1996; Divers & Peek, 2008).

It is known that the self-limiting character of the infection is mainly associated to 3 factors: the high regenerating capacity of the gut epithelium (3-6 days); the fact that the regenerated cells are almost resistant to new infections and that the immune system induces a local immunity at the previously affected site (Fayer *et al.* 1998; Kaske & Kunz, 2003).

The period of time necessary for the improvement of the clinical signs was probably related to the infectious doses, immunity of each patient, existence of concurrent infections, nutritional status and husbandry management, as these factors determine greatly the severity of the infection (Olson *et al*. 2004; Radostits *et al*. 2007; Coklin *et al.* 2009).

Most frequently encountered clinical signs in the affected calves, on the day of entrance in the clinic and diagnosis of cryptosporidiosis, were diarrhea (which varied between watery and thin mushy), dehydration (varying from slight to severe) and, in some cases, weakness, hypothermia and reduced feed intake. These findings correlate with the data in current literature, which state the most common signs of a *Cryptosporidium* infection as diarrhea, dehydration, abdominal pain, reduced appetite and depression (de Graaf *et al.* 1999b; Naciri et *al.* 1999; Olson *et al*. 2004; O'Handley & Olson, 2006; Divers & Peek, 2008; Kváč *et al.* 2011).

Hypothermia, due to lack of energy and dehydration, which is a common finding in cases of diarrhea (Kaske & Kunz, 2003), was, however, only detected in 2 cases (patients no. 7 and 8).

In most cases, the diarrhea was watery (patients 1, 3, 4, 5) or watery to thin mushy (patients 7 and 8) and yellowish. This finding makes us partly agree with several sources, which state that the characteristic diarrhea of cryptosporidiosis is profuse and persistent watery, yellow-greenish colored with putrid smell and containing mucus (de Graaf *et al.* 1999b; Rommel *et al.* 2000; Olson *et al*. 2004; O'Handley & Olson, 2006; Radostits *et al.* 2007; Thompson *et al.* 2008).

The most frequently encountered alterations in the laboratory analysis were slight to severe metabolic acidosis (in 6 out of 8 calves), an increase of lactate (4 out of 8 calves) and hyponatremia (4 out of 8 calves). Several publications state these findings as common in cases of diarrhea: metabolic acidosis is common due to loss of bicarbonate, retention of hydrogen ions and formation of organic acids (Kaske & Kunz, 2003; Koch, 2004; Lorenz, 2009; Smith, 2009); lactate isomers are increased due to bacterial fermentation (Lorenz, 2004; Berchtold, 2009; Lorenz, 2009; Smith, 2009) and sodium levels tend to be low due to losses in the feces (Michell *et al.* 1998; Kaske & Kunz, 2003; Koch, 2004).

As a side-note, it is important to emphazyse that the performed lactate measurement at the clinic could only detect L-lactate. As D-lactate seems to be even more important in cases of diarrhea, lactate increase might be underestimated (Rollin *et al.* 2006; Lorenz, 2009).

Other lab findings included hyperkalemia (n=3), slight alkalosis (n=2), leukocytosis (n=2), polycitemia (n=1), hypoglycemia (n=2) and increase in urea and creatinine (n=1).

Hyperkalemia is a paradox finding in cases of diarrhea as usually diarrheic calves show low serum levels of potassium due to losses in the feces and activation of aldosterone (Michell *et al.* 1998; Koch, 2004; Smith, 2009). The increase of the serum potassium might be caused by the impaired renal excretion caused by dehydration or it can be also related to

malfunctions of the Na^+- K^+- ATPase pump due to metabolic acidosis, as it only functions well in neutral pH (Berchtold, 2009; Smith, 2009). This causes intracellular accumulation of Na^+ ions and increase of K^+ in the extracellular space (which usually contains low concentration of potassium). In the end, this process leads to hyperkalemia, even though intracellular and total body concentrations of potassium are low (fecal loss) (Berchtold, 2009; Smith, 2009).
In rare cases, calves with diarrhea can show metabolic alkalosis. However, the reason for this is not fully understood to this date (Kaske & Kunz, 2003).
Some calves in our study group did not show any pH changes. This can be confirmed by a study conducted by Vos *et al.* (2005) which showed surprising results as no major pH alterations (metabolic acidosis) were detected in the examined calves.
Leukocytosis was present possibly due to the immune stimulation by *Cryptosporidium* or other infections (de Graaf *et al.* 1999a; Gookin *et al.* 2002; Tzipori & Ward, 2002).
Relative polycitemia due to dehydration and consequent hemoconcentration is also a common finding in cases of diarrhea (Michell *et al.* 1998; Koch, 2004; Berchtold, 2009).
Reduction in blood glucose due to the reduced feed intake because of the inappetence, the decreased gut absorption and neoglucogenesis, the higher glucose metabolism and the presence of inflammatory factors and endotoxins due to septicemia are also present in diarrhea cases (Kaske & Kunz, 2003; Koch, 2004). The low occurrence in this study is probably related to the entrance and treatment of the calves in the beginning of the affection, being the occurrence of hypoglycemia more frequent in chronic diarrhea cases and when septic conditions are established.
The increases in urea and creatinine can also be explained as a consequence of dehydration, reduced kidney perfusion and accumulation of metabolic products (Kaske & Kunz, 2003).

Mixed infections were evidenced in two cases. Patient number 2 was infected by *Cryptosporidium* and *Coronavirus* and patient number 5 was infected by *Cryptosporidium* and *Rotavirus*. Probably some mixed bacterial infections existed, however, as most calves were pretreated with antibiotics and the bacterial culture would not be representative, no bacterial determination was performed in our cases. More cases of mixed infections involving viruses could be present but no longer detected, as viruses can only be evidenced in the first few days after the start of the diarrhea symptoms (Bopp, 2003).
Mixed infections in cases of cryptosporidiosis are frequent (de la Fuente *et al.* 1999; Naciri *et al.* 1999; Radostits *et al.* 2007). Against the expected facts however, which state that mixed infections show worse clinical signs and prognosis (higher acid-base and electrolyte imbalances, dehydration and possible dysentery) (Divers & Peek, 2008), the clinical signs evidences by the calves number 2 and 5 were not more intense, nor the health situation and prognosis less favorable when comparing to the others.

De la Fuente's *et al.* (1999) results showed that *Rotavirus* was the agent that most often appeared in combination with *Cryptosporidium* and appeared most frequently in the calves' first week of life. Following Blume's results (2007) this combination was considered to be responsible for the highest mortality rate due to diarrhea in calves.

Even though this study can not confirm this finding due to the low number of animals, calf number 5 showed a mixed infection of *Cryptosporidium* and *Rotavirus*.

The degree of diarrhea in this study does not seem to be related to the severity of *Cryptosporidium* oocyst excretion. For instance, calf number 8 evidenced profuse watery diarrhea and was highly acidotic but was only classified as + regarding the oocyst excretion, while calf number 6 had very transient thin mushy (more consistent) diarrhea and a slight acidosis and was classified as +++ in behalf of the oocyst excretion.

Regarding the treatment, it focused mainly on oral and intravenous fluid therapy (for hydration, sodium and chloride increase), treatment of acidosis with sodium bicarbonate (intravenously or orally) and antibiotherapy to avoid septicemia (preferably with Cobactan®). Halocur® was not consistently used and as the treated calves did not show better evolution when comparing to calves not treated with Halocur®, its effect is not sure to be entirely efficient in the improvement of clinical signs.

Several studies showed that halofuginone is not entirely effective. Only in some studies it showed positive results (Villacorta *et al.* 1991; Jarvie *et al.* 2005; Klein 2007), while in others there was seen no significant improvement of the calves' health status (Naciri *et al.* 1992; Peeters *et al.* 1993).

Oral rehydration was administered several times a day through a nipple. This way the hydration and electrolyte balance was reestablished by allowing the passage directly to the abomasum (activation of the esophageal groove) with a consequently fast absorption (Naylor et al. 1999; Kaske et al. 2003).

The administration of bicarbonate *per os* in form of a tablet (Bi-Pill®) was scheduled in between the milk-feeding with a sufficient interval to allow milk-clotting (Michell *et al.* 1998; Naylor *et al.* 1999; Constable *et al.* 2001; Kaske & Kunz, 2003; Nappert & Spennick, 2003; Naylor *et al.* 2006).

Feeding milk replacer fluid was continued throughout the treatment period in all calves. As already stated in the literature review, the interruption of the milk replacer may have deleterious effects in calves: higher weight loss and a negative energy balance (decreased glucose, increased β- OH- butyrate and nonesterified fatty acid concentration) (Brooks *et al.* 1996b; McClure, 2001; Smith, 2009). Also, milk feeding might favor the regeneration of the gut mucosa (McClure, 2001; Kaske & Kunz, 2003; Constable *et al.* 2009) and there might exist a possible specific inhibiting effect the milk lipid fraction exerts upon the adhesion of the parasite to the host cell (Johnson, Schmidt, Gelberg & Kuhlenschmidt, 2004; Constable *et al.* 2009).

In this case, Milli® from Sano was used, which consists of a colostral milk replacer enriched with carotin (Sano - Moderne Tierernährung GmbH, 2011).

Disinfection is a crucial point in the control of cases of cryptosporidiosis (de Graaf *et al.* 1999b; Kaske & Kunz, 2003). In this case disinfection was performed by the use of two different products: Venno® Vet - 1 and Neopredisan® 135 – 1.

Venno® Vet- 1 contains several organic acids and has a spectrum of disinfection on virus with or without envelope, fungi and bacteria (MENNO CHEMIE-VERTRIEB GMBH, 2003). Cryptosporidium oocysts do not seem to be destroyed by this product, showing their great resistance towards desinfectants in comparison to other microorganisms (Rommel *et al.* 2000; Bopp, 2003; Kaske & Kunz, 2003).

Neopredisan® 135-1 has p-Chloro-m-cresol as its active ingredient. As it acts on wormeggs, coccidia, cryptosporidia, several kinds of bacteria, fungi, virus, spores of clostridia, tuberculosis and prions, it seems to be more potent than the previously refered (MENNO CHEMIE-VERTRIEB GMBH, 2009).

Successful disinfection to avoid cryptosporidiosis is, hence, mainly attributable to Neopredisan® 135-1.

Personal protection was sensibilized towards the use of disposable gloves and special aprons for the section, combined with disinfection after the handling of the calves. This goes in accordance with Harp & Goff (1998), who described possible personal protection and its significance in cases of cryptosporidiosis.

This personal protection was done to avoid cases of zoonosis and public health concerns. However, some zoonotic cases were detected nevertheless, probably mainly in more immune compromised individuals (Fayer *et al.* 1998).

Levine, Levy, Walker and Crittenden (1988) studied the incidence of *Cryptosporidium* infection in veterinary students involved in the handling of infected calves. At least 10 of the 72 students were excreting oocysts and developped clinical signs, 5 to 14 days after exposure, including fever, headache, nausea, diarrhea and/or vomiting. One affected student even required hospitalization due to the high degree of dehydration, even though he showed no signs of immune depression prior to the infection. Emphasis was given to the correct hygienic and sanitary practices.

Conclusion:

Following this small study, we can conclude that the most commonly detected disorder in the calves was diarrhea and, regarding those, a high proportion excreted *Cryptosporidium* oocysts (36%). The parasite is present and moderately prevalent in the region of the present study (Hessen, Germany), in calves aged 2 to 14 days.

Clinical signs most often shown by the calfs included watery to thin mushy diarrhea, dehydration, weakness, hypothermia and reduced feed intake, in different degrees.

On a laboratory basis, metabolic acidosis, increase of lactate and hyponatremia were most often present; followed by less frequently encountered findings, such as hyperkalemia, slight alkalosis, leukocytosis, polycitemia, hypoglycemia and increase of urea and creatinine.

Mixed infections were evidenced and are known to exist. However, mixed infections by *Rotavirus* or *Coronavirus* did not show worse clinical signs and outcome than monoinfections with *Cryptosporidium* spp.

The severity of diarrhea was not considered to be correlated with the amount of excreted oocysts.

Most important treatment was considered to be the fluid therapy, control of acidosis and the antibiotherapy, while the administration of Halocur® (halofuginone lactate) is not a sure improvement in the treatment.

Summing up it can be concluded that *Cryptosporidium* is present and prevalent in Hessen, Germany alone or together with other infective agents, causing more or less pronounced clinical signs and having usually a good prognosis if the treatment (especially the fluid therapy and alkalinization), disinfection and colostral management are done in an adequate way.

Case Study in the Ribatejo area, Portugal:

Introduction:

In the Summer of 2011 (July to September), 30 calves from a fattening unit in the Ribatejo area were randomly selected, clinically examined and fecal samples taken.

Purposes:

The purposes of this second study were:

- To evaluate the prevalence of cryptosporidiosis in calves in the Ribatejo area, Portugal, by detection of oocysts in the feces.
- The listing of the most apparent clinical signs and correlating them with positive detections of oocysts.
- Evaluation of the relationship between the housing (inside and outside) and the clinical signs and positivity to cryptosporidiosis.

Materials and Methods:

On the 18th July 2011, a fattening unit located in the Ribatejo area near Lisbon, Portugal, was visited.

At a moment, 300 to 400 animals were housed in the unit, originary from several farms located in the south of Portugal. A total of 30 animals were examined, around 7,5 to 10% of the population.

In July, fifteen calves in their first 2 weeks of life were randomly selected and generally examined for their most apparent clinical signs. Examined parameters included: Rectal temperature, hydration status, mucous membranes, heart rate, breathing rate, lung sounds and appearance of the umbilicus, the abdomen, the articulations and the feces. Moreover, housing systems were recorded in each calf. The calves were housed in 2 different areas, outside and inside.

From all 15 examined calves, fecal samples were taken in sterile containers (directly from the rectum and by using disposable gloves) and kept for further analysis in the parasitology laboratory from the Veterinary Faculty – Technical University Lisbon.

At the faculty's laboratory, the samples were first stored in a refrigerator (4 – 5 º C) during 48 hours and then analyzed by the modified Ziehl-Neelsen technique to discover the presence of oocysts.

Modified Ziehl-Neelsen technique:

1. A direct fecal smear was made, mixing well the feces in the containers and spreading a thin layer over a degreased microscope slide.
2. The smears were left to dry completely, over night.
3. All slides were covered with a layer of Metanol, during 1 minute.

4. Fuchsine was applied and left to act during 10 minutes.

5. The slides were washed with hydrochloric alcohol 1%.

6. Malachite green coloring 0,4% was applied on the slides for 30 seconds.

7. The slides were dried with absorbent paper, taking care not to scratch the surface.

8. The slides were observed under the light microscope with the 100x magnification objective and by applying immersion oil.

After analyzing the samples microscopically and searching for oocysts, the obtained data was analyzed qualitatively.

On the 16th August 2011, the fattening unit was visited again and the previously examined calves were reexamined to determine their evolution. Clinical signs were registered and analyzed.

Finally, on the 26th September 2011, the last visit took place and another 15 calves were examined and fecal samples taken. Microscopical observation at the parasitology laboratory of the Veterinary Faculty – UTL was performed and the obtained data was analyzed.

Statistical relationship between the presence of diarrhea and the presence of oocysts in the feces and the presence of diarrhea and dehydration was determined:

- The qualitative values were transformed into numerical variables by attributing “0” to the negative or absent findings (no diarrhea, negative Ziehl-Neelsen Test and absent dehydration) and “1” to the positive or present findings (presence of diarrhea, positive Ziehl-Neelsen Test and dehydration).
- Excel® 2007 was used as an analysis tool to determine correlation coefficients and the regression line.

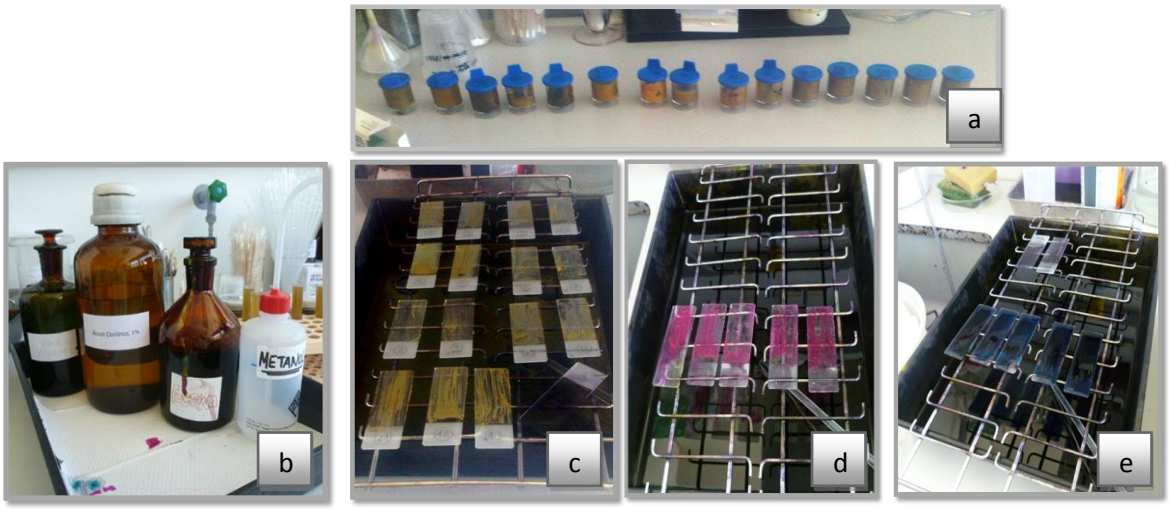


Fig. 16 - 20: Material and procedures for the modified Ziehl-Neelsen technique. Fecal containers (a). Solutions and reagents (Malachite green, hydrochloric alcohol, fuchsine, methanol) (b). Fecal smears (c). Slides after hydrochloric alcohol application (d). Malachite green application (e) (Original pictures).

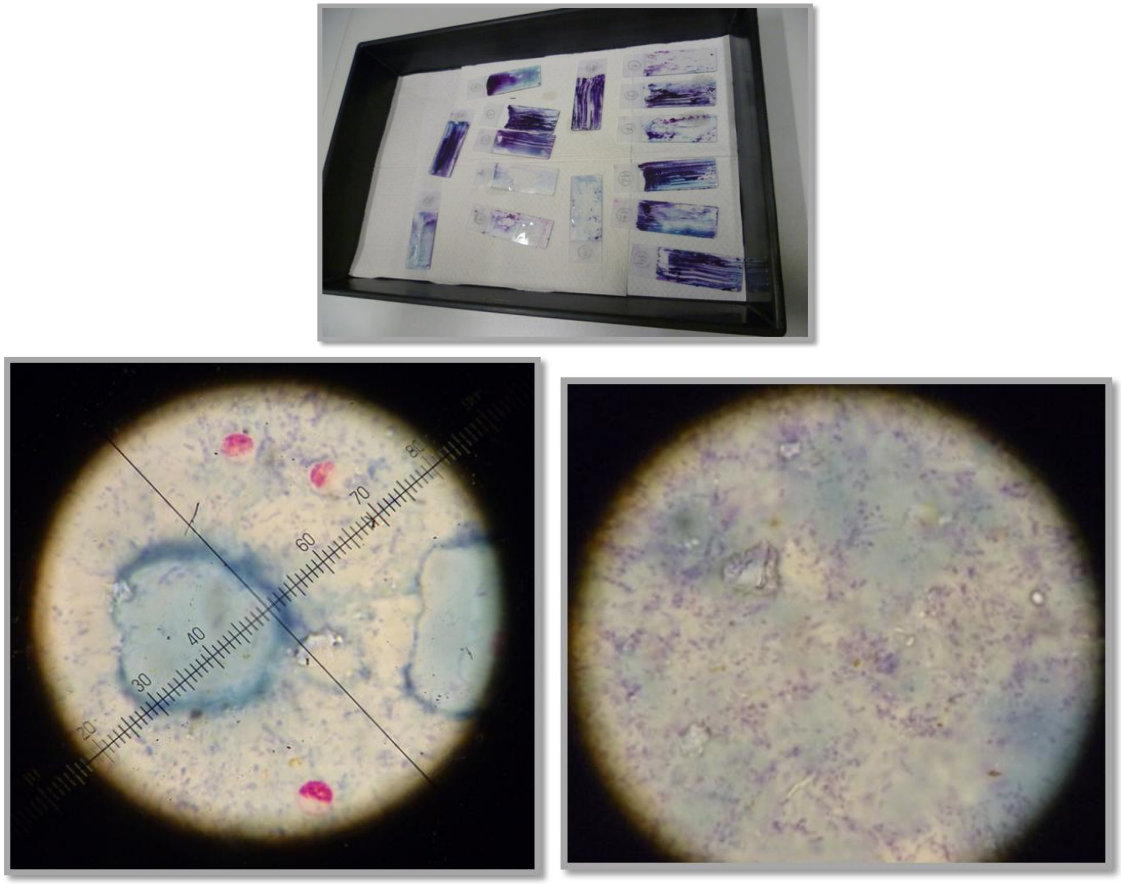


Fig. 21-23: Microscopical observation of the colored slides. Prepared slides (top).

Positive slides will show round pink formations in front of a green background. Magnification: 800 x (bottom left). Negative slides will show a green background with several structures in blue / purple, no pink formations are seen (bottom right) (Original pictures).

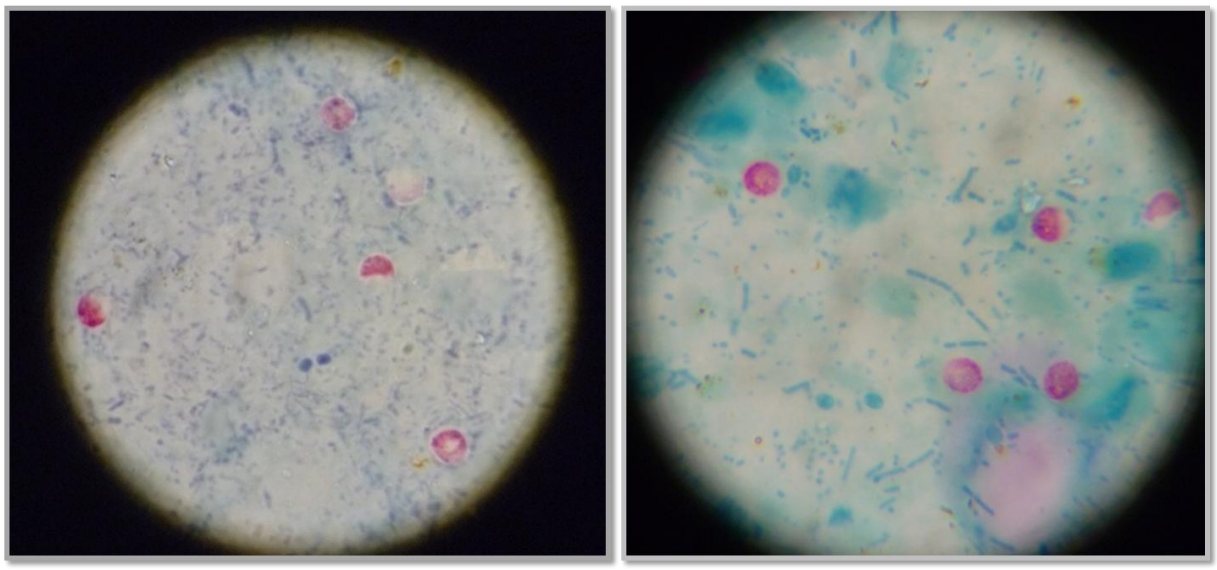


Fig. 24 and 25: Positive samples. Oocysts are highlighted in pink, in front of a greenish background (Original pictures). Magnification: 800 x (left) and 1000 x (right).

Results:

Calve:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Temperature (38,5 - 39,5ºC)	38,5	38,2	38	38,3	38,3	38,5	39,2	38,9	38,5	37,9	38,7	38,4	38,3	38,5	38,1
Heart Rate (80 - 120 bpm)	136	104	152	112	108	96	112	96	100	80	100	152	116	136	144
Hydration	Normal	Normal	Slightly dehydrated	Normal	Normal	Slightly dehydrated	Normal	Slightly dehydrated	Normal	Dehydrated	Normal	Normal	Normal	Normal	Normal
Mucous membranes	Normal	Normal	Pale	Pale	Normal	Pale	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Breathing Rate (20-40 vpm)	60	28	40	24	48	32	88	24	60	48	52	24	20	48	24
Lung sounds	Normal	Normal	Normal	Normal	Increased	Normal	Increased	Increased	Increased	Highly increased lung sounds	Normal	Normal	Normal	Normal	Normal
Umbilicus	Normal	Normal	Normal	Normal	Normal	Painful	Normal	Dilated	Normal	Normal	Normal	Normal	Hernia	Normal	Normal
Tympanic	Negative	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative
Articulations	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Swollen Carpal joints
Feces	Pasty/Mushy	Pasty	Thin mushy	Pasty	Pasty	Fluid	Gelatinous	Aquous	Pasty	Mucous	Mushy	Pasty	Pasty / Mushy	Mushy	Pasty
Diarrhea	No	No	Yes	No	No	Yes	No	Yes	No	Yes	No	No	No	No	No
Ziehl-Neelsen	Negative	Negative	Positive (+)	Positive ++	Positive +	Positive +++	Negative	Positive +	Positive ++	Positive +++	Positive (+)	Negative	Negative	Negative	Negative
Housing	Out	Out	Out	Out	Out	Out	Out	Out	Out	Out	In	In	In	In	In

Altered clinical signs / findings.

Table 3: Results of the first examination of 15 randomly selected calves from the ribatejo area (18. July 2011).

Additional Observations: The general body condition of the majority of the calves was acceptable to good. An exception constituted the calves with the numbers 6, 8 and 10, who showed significant signs of weakness (poor standing ability) and weight loss. The calves with the numbers 6 and 10 had lesions on the posterior part of the body: hind legs and perianal area, with presence of larvae (myasis).

Calve:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Temperature (38,5 - 39,5ºC)	38,6	38,6	38,3	39	38,7		38,6		38,9		39,5	39,7	38,7	38,9	38,3
Heart Rate (80 - 120 bpm)	108	146	160	96	84		108		100		96	72	76	104	100
Hydration	Normal	Normal	Slightly dehydrated	Normal	Normal		Normal		Normal		Slightly dehydrated	Slightly dehydrated	Normal	Normal	Slightly dehydrated
Mucous membranes	Normal	Normal	Normal	Pale	Normal		Normal		Normal		Pale	Normal	Pale	Normal	Pale
Breathing Rate (20-40 vpm)	56	48	52	24	56		40		48		40	88	28	24	32
Lung sounds	Normal	Slightly increased, coughing	Slightly increased sounds	Normal	Slightly increased		Slightly increased		Slightly increased		Normal	Highly increased sounds	Normal	Normal	Normal
Umbilicus	Normal	Normal	Normal	Normal	Hard, no pain		Normal		Normal		Normal	Normal	Swollen and Painful	Normal	Normal
Tympanic	Negative	Negative	Negative	Negative	Positive		Negative		Negative		Negative	Negative	Negative	Negative	Slightly Positive
Articulations	Normal	Normal	Normal	Normal	Carpal joints slightly swollen		Normal		Normal		Normal	Left Carpus swollen	Normal	Normal	Normal
Feces	Pasty	Mucous	Pasty	Pasty	Thin Mushy		Pasty		Pasty		Pasty	Pasty	Mucous	Mushy	Pasty
Diarrhea	No	No	No	No	Yes		No		No			No	Yes	No	No
Ziehl-Neelsen															
Housing	Out	Out	Out	Out	Out		Out		Out			In	In	In	In

Altered clinical signs / findings. Deceased Disappeared / no Data

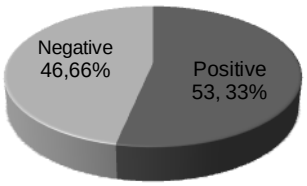
Table 4: Results of the second examination of the previously examined calves from the Ribatejo area (16th August 2011).

Calve:	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Temperature (º C) (38,5 - 39,5ºC)	38,6	39,3	38,4	38,9	38,1	38,4	39,6	39,5	40,6	38,8	38,3	38,8	39,2	39	39
Heart Rate (bpm) (80 - 120 bpm)	112	92	76	96	104	92	120	112	132	124	88	120	120	144	164
Hydration	Normal	Normal	Normal	Normal	Slight dehydration	Normal	Slight dehydration	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Mucous membranes	Pale	Normal	Normal	Reddened	Pale	Pale	Normal	Pale	Normal	Normal	Normal	Normal	Normal	Pale	Normal
Breathing Rate (vpm) (20-40 vpm)	32	36	24	48	44	44	40	56	52	64	36	56	40	96	44
Lung sounds	Normal	Normal	Normal	Increased, seromucous nasal discharge	Slightly increased	Normal	Normal	Slightly increased	Normal	Normal	Normal	Normal	Normal	Highly increased	Normal
Umbilicus	Normal	Slight Hernia	Normal	Swollen, warm, firm	Slightly swollen, firm	Swollen, warm, firm	Hernia	Hernia	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Tympanic	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Slightly Positive	Negative	Negative	Negative
Articulations	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Feces	Pasty	Pasty	Slimy, traces of blood	Mushy	Gelatinous	Pasty	Mushy	Pasty	Mushy	Pasty	Pasty	Pasty	Thin mushy	Gelatinous	Mucous
Diarrhea	No	No	No	No	Yes	No	No	No	No	No	No	No	Yes	No	Yes
Ziehl-Neelsen	Negative	Negative	Negative	Negative	Positive +++	Negative	Negative	Positive +	Negative	Negative	Negative	Positive (+)	Positive (+)	Negative	Positive (+)
Housing	Out	Out	Out	Out	Out	In	In	In	Out	Out	Out	Out	In	In	In

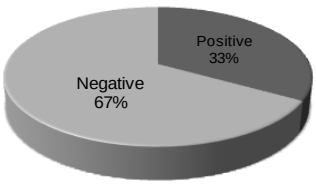
Altered findings/ clinical signs.

Table no. 5: Results of the second group of randomly selected calves from the Ribatejo area in Portugal (26th September 2011).

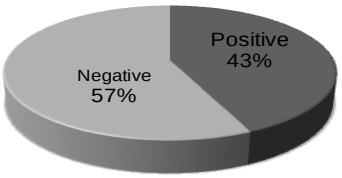
Prevalence:



Graph. 3 : Prevalence of the excretion of oocysts in the first 15 examined calves.



Graph. 4 : Prevalence of the excretion of oocysts in the second group of 15 calves (calf 16 to 30).



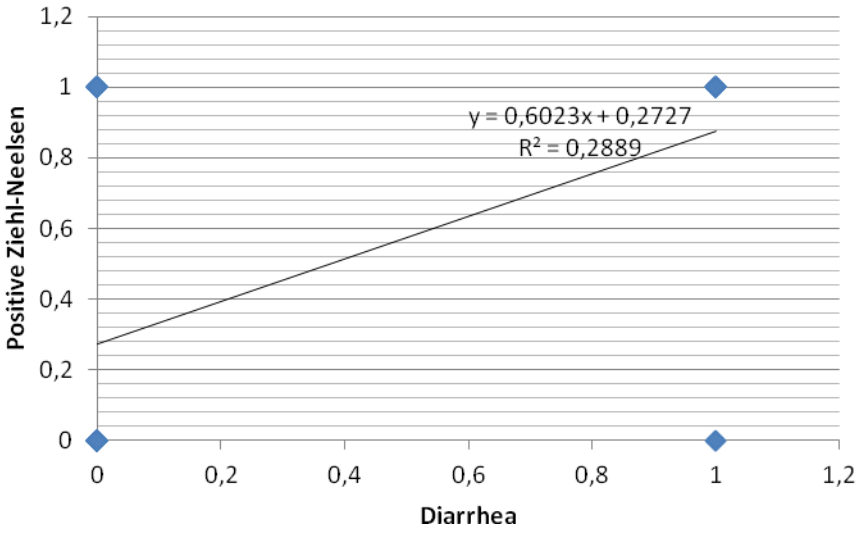
Graph. 5: Overall prevalence of the excretion of oocysts in the 30 tested calves.

Correlation and regression:

Testing the relationship between the presence of diarrhea and the detection of oocysts (positive modified Ziehl-Neelsen test) in the 30 tested calves:

Correlation coefficient (r) = 0,537

Linear Regression:

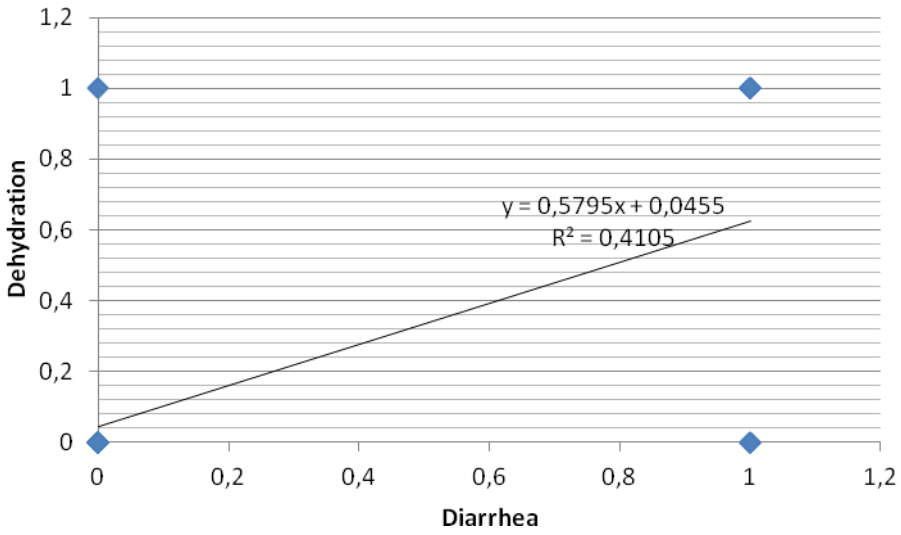


Graph. 6: Linear regression between the incidence of diarrhea and a positive modified Ziehl-Neelsen result.

Testing the relationship between the presence of diarrhea and of dehydration in the calves:

Correlation coefficient (r) = 0,641

Linear regression:



Graph 7: Linear regression between the presence of diarrhea and the presence of dehydration in the analyzed calves.

Fig. 26 and 27: Housing systems from the examined fattening unit in the Ribatejo area. Outside housing (left) and Inside housing (right) (Original picture).

Discussion:

Taking our previously established objectives into account, the calves were examined for their general health condition and fecal samples were taken from each animal for further analysis. Taking the principles of the modified Ziehl-Neelsen technique into account, oocysts are stained in pink as this parasite is considered to be an acid-fast structure (Fayer *et al*. 2000; Rommel *et al*. 2000; Tzipori & Ward, 2002). Carbolfuchsin is easily taken up by the lipoid

capsule of acid-fast organisms, staining them in bright pink. This capsule is resistant to the washing procedure with hydrochloric acid, as well as to the coloration with water soluble stains (such as malachite green). This is why the oocysts appear in pink and the background in green in the modified Ziehl-Neelsen technique (Allen, 1992; The Internet Pathology Laboratory for Medical Education, 2010).

The analysis in the laboratory showed the presence of 8 positive samples from the 15 first samples taken on the 18th July 2011. In the second group of analyzed calves, however, only 5 of the 15 samples were positive. The overall prevalence of *Cryptosporidium* infection in the fattening unit was of 43,33 %. This prevalence can be considered as rather high as almost half of the analyzed calves excreted oocysts.

In a previous study in Portugal (in the north) the detected prevalence of cryptosporidiosis in calves was close to 25 % (Mendonça *et al.* 2007).

In the Ribatejo area and the Alentejo, studies by Fonseca (2000) determined a global prevalence of 23,3% of cryptosporidiosis (21,1% in the Alentejo and 34% in the Ribatejo area). A slightly higher prevalence was detected in the calves aged 16 to 24 days (47,2%) when comparing to the 8 to 15 day old calves (41,7%).

The species of *Cryptosporidium* that constituted the infecting agent could not be determined as the diagnosis was made by microscopical observation (which is unable to differentiate the different species). The specific determination could be only attained by molecular analysis and determination of the genotype (Tzipori & Ward, 2002; Divers & Peek, 2008).

However, following results of the most prevalent *Cryptosporidium* species in calves in their first 2 weeks of life, *Cryptosporidium parvum* would be the most probable species (Mendonça *et al.* 2007; Divers & Peek, 2008; Imre *et al.* 2011; Kváč *et al*. 2011; Muhid *et al.* 2011).

Analyzing the overall appearance of the calves in the first general examination, following points could be detected:

- All calves that had diarrhea also excreted *Cryptosporidium spp.* oocysts.
- However, not all calves that excreted *Cryptosporidium* spp. oocysts had diarrhea.

These two points highlight the role of *Cryptosporidium* spp. in originating signs of diarrhea, while excretion can persist after or without the appearance of this clinical sign. This is supported by current literature (de Graaf *et al*. 1999; Naciri *et al*. 1999; Olson *et al*. 2004; O'Handley & Olson, 2006; Divers & Peek, 2008; Kváč *et al*. 2011).

The statistical analysis found a moderate correlation between the incidence of diarrhea and the presence of oocysts in the feces (positive to modified Ziehl-Neelsen).

It is important to remember that a correlation coefficient of 1 or -1 indicates a perfectly linear and a complete positive or negative relationship, respectively. If the correlation coefficient is equal to 0 there is no linear relationship between the two variables (Stockburger, 1998).

Taking into account that: the higher the correlation, the closer to |1| the value of the correlation coefficient; we can consider that the correlation coefficient value of 0,537 indicates a moderate correlation between the two variables.

This finding, together with the regression line, indicates that diarrhea and the positivity of the modified Ziehl-Neelsen test do not always coexist.

The studies from Kváč *et al.* (2011) were only confirmed partially. Their results indicated that *Cryptosporidium* infection is more frequent in cases of diarrhea than if the feces have pasty consistency. In the present study, however, only around half of the infected animals (7 out of 13) had diarrhea, while the others (6 out of 13) had normal fecal consistency.

The regression line was drawn merely to illustrate this relationship, no future predictions can be made, as the variable values could only take the values 0 and 1.

- Calves with signs of diarrhea had some degree of dehydration.
- Only a few cases were dehydrated and had no diarrhea.

These findings show the correlation between the presence of diarrhea and the loss of fluids and electrolytes, which is known to exist (Michell *et al.* 1998; Berchtold, 2009).

This correlation was confirmed in the statistical analysis, as the correlation coefficient between the two variables (diarrhea and dehydration) was moderate to high (r = 0,641) and by the observation of the regression line. The correlation is not perfect, though, which suggests the existence of other dehydration causing mechanisms or diseases.

- The severity of diarrhea and dehydration was not related to the amount of oocysts that were excreted in the feces. Calves with more fluid feces were not necessarily infected to a higher degree. For instance, Calve no. 3 had diarrhea but excreted a small amount of oocysts ((+)), while calf no. 4 had no diarrhea but excreted a higher number of oocysts (++).

This finding is, though, rather subjective and error-prone. Determination of the intensity of the infection by the attribution of one or several “+” signs did not follow any scientific standards and can not be considered a reliable measure of the intensity of infection. It reflected somehow the time spent for the determination of the sample as positive and the number of oocysts detected in each microscopic optical field.

Regarding the feces of the animals that were positive for cryptosporidiosis, feces that were non diarrheic could show more positivity because they were less diluted. Animals with diarrhea probably would be more severely affected but the number of detected oocysts would be lower, as the feces were more fluid (containing more water) and, hence, more diluted.

It is of crucial importance, though, that in the case of the calves with the numbers 6 and 10, the intensity of diarrhea, excretion of oocysts and dehydration were closely related. They constituted the most severely affected calves and demonstrated that fact clearly by their poor general condition, the severity of diarrhea and their hydration status.

- Calves infected with cryptosporidiosis were often also suffering from diseases in other organs (E.g. lungs and umbilicus). I. e. 16 of the 30 tested calves showed alterations in other organs (lungs: no. 5, 7, 8, 9, 10, 19, 20, 23, 29; umbilicus: no. 6, 8, 13, 17, 19, 20, 21, 22, 23; tympany: 3, 5, 14, 27). Nine of these 16 calves (56 %) were excreting *Cryptosporidium* oocysts and also, from the 13 positive calves, 9 (no. 3, 5, 6, 8, 9, 10, 20, 23, 27) (69 %) showed clinical signs which suggested the affection of other organs.

These findings suggest that immune depression by other diseases can trigger infection by cryptosporidiosis, as well as that cryptosporidiosis is a debilitating disease that can predispose calves to other diseases. This finding was also discovered previously, and is documented in several scientific documents (de la Fuente *et al*. 1999; Naciri *et al*. 1999; Kaske & Kunz, 2003; Radostits *et al*. 2007).

But this was not completely linear, as calves affected by cryptosporidiosis sometimes had no other organs affected, while some calves that were negative to cryptosporidiosis, showed clinical signs suggesting lesions in other organs.

- Pale mucous membranes (which suggest an anemic state) were predominantly present in calves that were excreting the parasite (no. 3, 4, 6, 20, 23). The calves with the numbers 16, 21 and 29 had signs of anemia but did not excrete oocysts.

This suggests that anemia can weaken the calves and increase the incidence of infections (including by *C. parvum*). However, not all calves that excreted the parasite, had anemia.

- No marked difference between the housing systems in respect to the incidence of cryptosporidiosis was detected.

While 47% of the calves that were housed outside (9 out of 19) were excreting oocysts, 54 % of the calves that were housed inside (6 out of 11) demonstrated oocysts in their feces. These results can be compared to the study by Kváč *et al.* (2011), in which no significant difference of incidence when considering the different housing systems was detected.

However, this finding goes against the current belief that the outside keeping of calves is preferred, as it provides better ventilation and lower concentration of infectious agents (Kaske & Kunz, 2003).

It is important to emphasize that the sample size was not representative and a higher number of calves housed outside were examined. Hence, these results do not constitute a reliable fact.

- Temperature was almost always in the normal range. Marked hypothermia was not a common finding, even though it is usually highly related to diarrhea and dehydration (Kaske & Kunz, 2003). One explanation could be the collection in a warm month of the year (July) in a southern country (Portugal), which are conditions that do not predispose the calves to hypothermia.

The first 15 calves were reexamined 1 month after the first examination (on the 16th August 2011) to evaluate the evolution of the clinical signs. Cross-relating the findings from the first and the second examination, the results showed marked variations.

The calves with the numbers 6 and 10, which showed the highest excretion of oocysts, poorer general body condition and severe diarrhea on the first visit, died between the two observation periods. The results show the possible relationship between the affection with *Cryptosporidium* and the death of calves in their first weeks of life. This data was confirmed by a study from Naciri *et al.* (1999), who found a relationship between the affection with *Cryptosporidium* and the death of the examined calves. In their study, from the 12 deceased calves, 10 were infected with *Cryptosporidium* spp.

As the sole infection with *Cryptosporidium* spp. is usually self limiting and mortality is significantly higher in calves suffering from mixed infection, the considered calves (no. 6 and no. 10) could be infected by other infectious agents besides *Cryptosporidium* spp. (Kaufmann *et al.* 1996; Kaske & Kunz, 2003; Divers & Peek, 2008). However, the collected samples were only tested for the presence of *Cryptosporidium* oocysts, so no conclusions can be made regarding the presence of other disease causing agents.

No data regarding the calf with the number 8 could be found on the second visit to the fattening unit. Due to its weakness, diarrhea and dehydration, it is probable that the calf died between the first and the second visit (like the calves with the numbers 6 and 10) but the data recording was deficient. Another possible cause might be a loss of the ear tags.

The other positive animals (3, 4, 5, 9 and 11) evolved in different directions. While the animals with the numbers 3, 4 and 9 showed marked improvement of the clinical signs showing the self-limiting character of this parasitic infection (Kaufmann, 1996; Fayer *et al.* 1998; Kaske & Kunz, 2003; Divers & Peek, 2008), the animals with the numbers 5 and 11 showed worsening of the clinical signs.

The calf with the number 5 developed signs of diarrhea and even showed affection of the articulations, while the number 11 developed fever, anemia and dehydration. These findings suggest that the animals were infected shortly after the first examination and fecal sample collection, and developed clinical signs related to cryptosporidiosis and concurrent infections in the following days. However, as this can not be proved, no conclusions can be made in this regard.

Animals that on the first examination were not excreting oocysts either maintained their health status (no. 1, 7, 14) or worsened (no. 2, 12, 13, 15). As no other examinations were made, there is no available data that allows the conclusion of any facts regarding the cause of the displayed clinical signs.

At the second visit, no fecal samples were analyzed for the presence of Cryptosporidium oocysts. The reasons for this was the low incidence of cryptosporidiosis cases due to *Cryptosporidium parvum* in calves with ages older than 4 weeks (Rommel *et al.* 2000;

Hamnes *et al.* 2006), coupled with the unavailability of the parasitology laboratory at the second examination period (16th August 2011).

The heart rates and breathing rates were not found to be related to the incidence of cryptosporidiosis. Both findings were often increased and might be related to the stress caused by the physical examination. High breathing rate was sometimes associated to respiratory diseases, however, as no other diagnostic measures were executed, no conclusions can be made in this respect.

Also, regarding the sex of the animals, no analysis was made, as the great majority of the examined calves were male (26 out of 30). In the study from Fonseca (2000) the incidence was slightly higher in females in younger calves (8 to 15 days of age), however, in calves aged 25 to 32 days the incidence was higher in males. These findings suggest that no sex predilection exists in cryptosporidiosis.

It is important to emphasize that the size of the sample was not representative of the whole population. It merely serves as an illustration of the reality of the disease in concrete cases.

Moreover, the sample collection was not performed in a completely random way. Considering the first group of 15 calves, those with worse health status and diarrhea were preferably chosen and examined for the study.

As the calves from the fattening unit were originary from several farms from the south of Portugal, the detected prevalence might not be representative for the Ribatejo area. The calves could have been infected in the farms of origin and carried the parasite to the fattening unit. Therefore, the obtained results may show the existence of cryptosporidiosis in several farms from the south of the country.

Conclusion:

In the present study, the detected prevalence for cryptosporidiosis was high. Almost half of the examined population excreted oocysts (43,33%). We, hence, can conclude that infections with this parasite are present and prevalent in the analyzed area (Ribatejo, near Lisbon and the south of Portugal).

No conclusions regarding the species affecting the calves could be made as the diagnostic method (modified Ziehl-Neelsen) did not allow species determination.

Often the excretion of oocysts came alongside with the existence of diarrhea. However, also assymptomatic carriers that excreted oocysts were detected and the statistical correlation between the two variables (presence of diarrhea and excretion of oocysts) was not very high (correlation coefficient = 0,537).

In cases where diarrhea was detected, it gave rise to cases of dehydration due to increased fluid losses. The associated correlation was moderately high (correlation coefficient = 0,641).

The incidence of diarrhea and dehydration were usually not accompanied by a higher number of excreted oocysts.

Two calves (number 6 and 10) had died between the first and second visit, which allows the conclusion that *Cryptosporidium* and its related consequences (alone or in combination with other affections) can be a cause of death in calves in their first weeks of life.
The self-limiting character of the disease was detected, as some calves markedly improved their clinical status at the second visit; while the coexistence of infections alongside with *Cryptosporidium* was found to have a high probability.
The analysis of the housing conditions did not allow for any conclusions as no significant differences between inside and outside housing were detected.
Summing up, *Cryptosporidium* spp. is currently present in calves from the Ribatejo area and the south of Portugal, non consistently giving rise to clinical signs and dehydration and being a possible cause of death in neonatal calves.

In general, with both clinical studies it was possible to confirm that *Cryptosporidium* exists and is prevalent in Germany and Portugal, in calves in their first 2 weeks of life.
Clinical signs included diarrhea, weakness, dehydration and hypothermia, but also assymptomatic carriers existed. Metabolic acidosis, increase in lactate and hyponatremia were the most important laboratory findings. Mortality can be increased by this agent, maybe together with concurrent infections.
Mixed infections with viral agents (*Rotavirus and Coronavirus*) can be detected, but are not always accompanied by worse clinical signs and outcome.
Fluidtherapy (through drip-infusion) and alcalinization, together with septicemia prevention through antibiotherapy seems to be the best therapy approach.
Disinfection and colostral administration should be always highlighted and are very important in the control of the disease. In order to decrease prevalence rates, these points should be constantly reviewed and improved in order to avoid losses due to cryptosporidiosis.

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Attachments:

Finding:	***Normal:***
Behavior	Calves are alert and curious.
Eating	Appetite is usually good and calves eat when feed is offered
Comfort	Comfortable calves are usually standing up when eating, lying down when resting
Stance	Calves usually stand squarely on their front feet
Heart rate	80 – 120 beats per minute Rythmic, strong and easily differentiated heart sounds
Breathing rate	20 – 40 breaths per minute Regular, no excessively loud breathing sounds (slight moist sounds after birth are normal, sounds are usually easier to hear than in adults)
Rectal temperature	38,5 – 39,5 º C
Mucous membranes	Pale pink. Capillary refill time < 2 sec.
Nose	Wet, cool to touch
Skin pinch	Skin should flatten in no more that 1 to 2 seconds (hydration status)
Eyes	Clear, no excessive tearing and no abnormal ocular discharge
Ears	Warm
Abdomen	Soft, not tympanic. Borborygmus in all quadrants.
Umbilicus	Dry, soft, not excessively warm, not painful
Articulations	Dry, not swollen, not painful
Urine color	Pale Yellow
Feces	Yellow and pasty, homogenous.

Attached table no. 1: Clinical findings that identify a healthy calf.

(Adapted from: Kaske M., Kunz H.-J. (2003). *Handbuch der Durchfallerkrankungen der Kälber* (pp. 15). Osnabrück, Germany: Kamlage Verlag.

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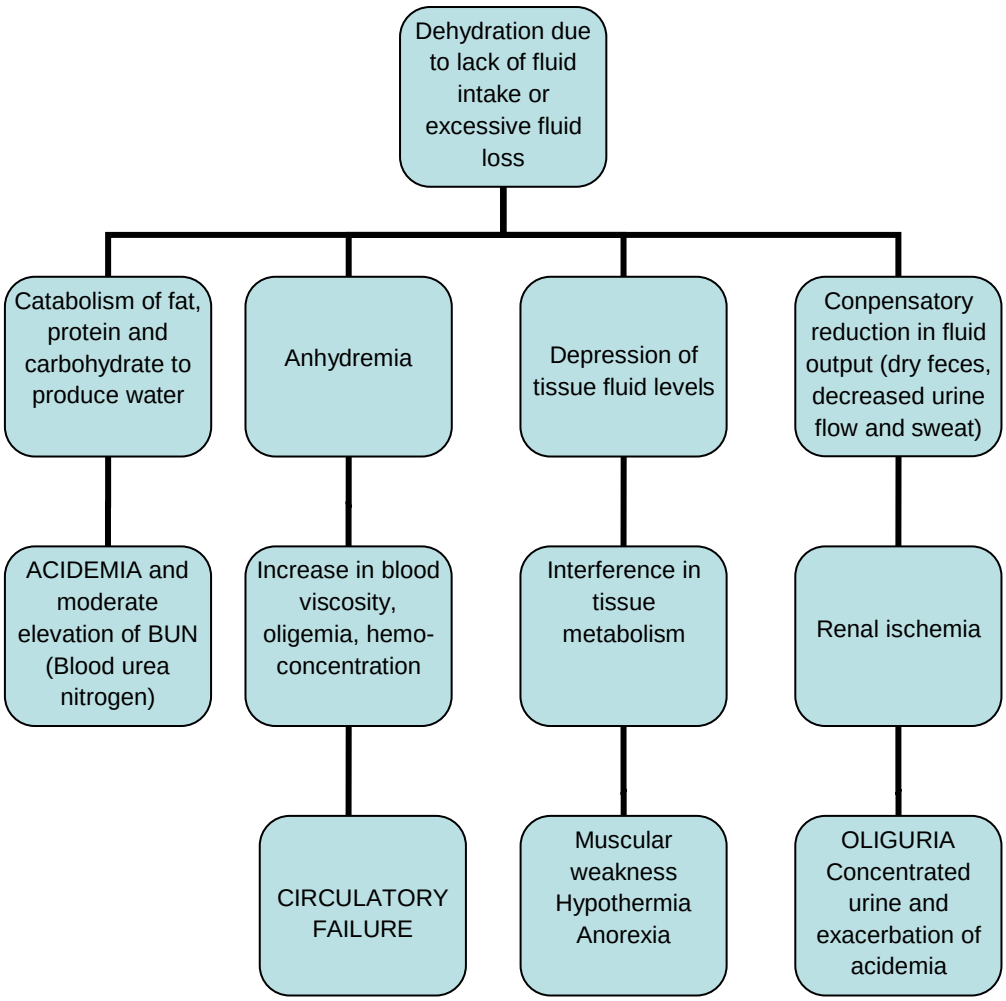

Attached graph. 1: Pathogenesis of dehydration.

In Radostits O. M., Gay C. C., Hinchcliff K. W., Constable P. D. (2007) Veterinary Medicine: A textbook of the diseases of cattle, horses, sheep, pigs, and goats (10th edition) (pp. 74). Philadelphia, USA.

Variable	Method of assessment	Score	Interpretation
Enophtalmus	Visual	1	Slight separation of eye from the eye lids
		2	Marked separation
Suckle reflex	Stimulated by putting the finger in the calf's mouth	0	Strong coordinated suckle movement
		1	Weak coordinated suckle
		2	Disorganized non productive suckle
		3	Absent
Menace reflex	Hand movement towards the eyes	0	Strong and instantaneous reflex
		1	Slow and delayed
		2	Absent
Tactile response	Pinch skin on lumbar area	0	Skin contraction and head movement towards the flank
		1	Only skin contraction, no movement of head
		2	No skin twitch or head movement
Ability to stand	Stimulate to stand (slightly spiking)	0	Can stand alone
		2	Can not stand unassisted
Warmth of oral cavity	Controling the temperature with the fingers	0	Normal warmth
		1	Cool mucosa
		2	Cold mucosa
Warmth of extremities	Tactile	0	Normal warmth
		1	Cool skin
		2	Cold skin

Attached table no. 2: Scoring system to determine the severity of metabolic acidosis in calves

(Adapted from: Kasari T. R. (1999) Metabolic Acidosis in Calves. Veterinary Clinics of North America: Food Animal Practice – Fluid and Electrolyte Therapy, 15, 478.)

Dehydration (%)	Clinical Signs
0	No enophthalmos; cervical skin-tent duration ≤ 2 s.; moist mucous membranes
2	Enophthalmos 1mm; cervical skin-tent duration 3 s; dry mucous membranes
4	Enophthalmos 2 mm; cervical skin-tent duration 4 s.
6	Enophthalmos 3 mm; cervical skin-tent duration 5 s.
8	Enophthalmos 4 mm; cervical skin-tent duration 6 s.; cool extremities.
10	Enophthalmos 6 mm; cervical skin-tent duration 7 s.; cold extremities.
12	Enophthalmos 7 mm; cervical skin-tent duration > 8 s.; cold extremities.
≥ 14	Enophthalmos >8 mm; cervical skin-tent duration > 10 s.; cold extremities; white mucous membranes.

Attached table no. 3: Hydration status evaluation.

In Berchtold J. (1999). Intravenous Fluid Therapy of calves. *Veterinary Clinics of North America: Food Animal Practice – Fluid and Electrolyte Therapy*. 15, 508.

Obtained from: Constable P. D., Walker P. G., Morin D. E. (1998) Clinical and Laboratory assessment of hydration status of neonatal calves with diarrhea. J. Am. Vet Med. Assoc. 212, 991 – 996.

Advance Arrest (MS Specialty Nutrition) [2]	Electrolyte HE (Pfizer) [2]	Life-Pak [1]
Biolyte (Pfizer) [2]	Electrolytes Concentrated (Austin) [1]	Mineralytes Oral Solution [1]
Bounce Back (Manna Pro) [2]	Electrolytes Concentrated (Dispar) [1]	OneBetter Calf electrolyte (Felton) [2]
Blue Ribbon Calf Electrolytes (Merrick) [2]	Epic calf electrolyte (Bioniche) [2]	Oralytes [1]
Bovine Bluelite C (Techmix) [2]	Hydra [1]	Oralytes HE [1]
Calf-lyte[1]	Hydrafeed (A&L Laboratories) [2]	Pro-lyte [1]
Calf-lyte II (Vetoquinol®) [1,2]	Hydralyte (Vet-A-Mix) [2]	Resorb (Pfizer®) [1,2]
Calf-lyte II HE (Vetoquinol®) [1,2]	Hysorb (Bimeda) [2]	Revibe (Wyeth®) [1,2]
Calf Quencher (Vedco®) [2]	Ion Aid ® [1]	Revibe® HE [1]
Deliver (Agri-Labs) [2]	Ionalyte[1]	Revitilyte (Vets Plus Inc.) [2]
Diaque (Boehringer Ingelheim) [2]	Life Guard HE [1]	Vita-Lytes (Vita Plus Corp.®) [1,2]
Electrate [1]	Life Guard Twin Pack [1]	V-Lytes HE [1]
Electrocarb-10 [1]		

Notes [1]:

- The mixing directions on the packages should be considered during the preparation, using warm rather than cold or hot water.
- Correct composition should consist of sodium at 70 to 120 mEq/l, chloride at 40-80 mEq/l and potassium at 10-20 mEq/l. Many products also contain an alkalinizing agent (40-80 mEq/l). Glucose, amino acids or other compounds facilitate the sodium uptake.

Attached table no. 4: Examples of oral rehydration solutions for calves as described in the Canadian Compendium of Veterinary Products[1] and available in North America[2].

From: [1] Ministry of Agriculture, food and rural affairs (2011) Electrolyte solutions for scouring dairy calves. Assessed on the 20th October 2011, available at: http://www.omafra.gov.on.ca/english/livestock/dairy/facts/electrol.htm

[2] Smith G. W. (2009). Treatment of Calf Diarrhea: Oral Fluid Therapy. Smith R. A., Smith G. S. (Editors). *Veterinary Clinics of North America. Food Animal Practice – Bovine Neonatology.* 25, 55-69. Philadelphia, USA: Elsevier Saunders. Page 58.

Correct placement of venous catheters:

To place an ear catheter, several steps have to be considered to facilitate the procedure and avoid contamination:

1) Shaving and disinfection (alcohol or iodine) of the site. A local anesthesia with Lidocaine is not advised to avoid difficult visualization of the vein. In rare cases, when the animal is still very active, xylazine sedation can be used (Berchtold, 2009).

2) Use a tourniquet (a rubber band or a straw band) on the ear base and, if necessary, put a humid and warm piece of gauze against the ear vein to increase its diameter (Berchtold, 2009; Kaske & Kunz, 2003).

3) For calves, usually a 22 gauge over-the-needle catheter is used, which is introduced in one of the ear veins (Berchtold, 2009). This catheter should have 0,9 mm diameter and 25 mm of length (Kaske & Kunz, 2003). There are several possible ear veins that can be used for catheterization: one or two cranial, one medial and one caudal ear vein. Before puncturing, make sure you are about to put the catheter in a vein and not in the artery that flows right besides. The artery is usually more prominent and can be seen before putting the tourniquet, it shows a pulse and its vessel wall is more rigid (Berchtold, 2009; Kaske & Kunz, 2003). When stroking the vein in direction to the ear base, keeping the finger pressed on it, it fills up again, while the artery remains empty (KGGA). In addition, if the artery is catheterized, the lighter colour of the blood alongside with the slower flow rate identifies it (Berchtold, 2009).

Attached fig. 1: Placement of a venous catheter. In Berchtold J., Prechtl J. (2003) Technik der Ohrvenen-Infusion beim Kalb. *Fachpraxis*. 43, Page 6.

4) Place the catheter carefully, checking, before advancing it in its full length, if there is blood in the catheter hub (correct vein placement) (Berchtold, 2009). As soon the catheter is in the vein, the mandril is pulled back slightly before advancing the catheter completely (Kaske & Kunz, 2003). The catheter should slide easily through the vein when correctly placed (Berchtold, 2009).

5) Fix the catheter with adhesive strips on the ear and connect the fluid administration line. Open the line completely to flush the catheter (alternatively with some heparinized saline) and fix the line on both ears with adhesive band (Berchtold, 2009). To warrant complete fixation of the catheter to the ear, it is possible to make

one or two stitches with an injection needle through the ear cartilage, and fix the catheter wings with rigid, monofilamentous suture material (Berchtold, 1999).

6) The infusion line is directed towards the basis of the ear in a hoop and fixed with more adhesive tapes. The infusion fluid container should be fixed 0,5 to 1,5 metres above the calf to allow the action of gravity. And the fluid line should be additionally fixed with an extensible dog leach, to allow the free movement of the calf by keeping the line stretched and avoiding any tangling up (Kaske & Kunz, 2003; Berchtold, 2009).

Catheterizing the jugular vein should be done by the following way:

1) The puncturing place on the upper third of the neck should be shaved, degreased and disinfected properly before introducing the catheter.

2) Catheters with 16 Gauge, 1,8 mm diameter and 45 mm length can be used and the catheter should be introduced towards the heart.

3) The catheter is fixed by suturing it to the skin of the neck. For this, a fold of skin is pinched under the catheter and a polyamide line introduced with a normal injection cannula. The catheter is then fixed with surgical knots.

4) In the end, the catheter has to be fixed additionally with adhesive tape and bandaging, and the infusion line should be fixed to the ear base again (Kaske & Kunz, 2003).

To guarantee the optimal supply of the intravenous rehydration therapy, the catheter should be correctly placed in the vein and conveniently secured. Perivascular infiltration should be avoided (Berchtold, 2009).

Attached fig. 2: Intravenous fluid therapy in a dehydrated calf as a continuous drip infusion (Original picture).

Patient No. 1: Female black Holstein calf. 2-3 days old.

Day / Parameter	1	2	3	4	5	6	7	8	9	10	11	12
Temperature (38,5 – 39,5º C)	38,9	39,1	38,8	38,9	39,2	38,3	39,7	39,1			39,0	38,0
Heart	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Hydration status	Slightly dehydrated	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Mucous membranes	Pale.	Normal.	Normal	Normal	Normal	Normal	Normal	Normal	Normal.	Normal	Normal	Normal
Lung sounds:	Normal	Normal	Normal	Normal	Increased	Increased	Slightly increased	Slightly increased	Slightly increased	Normal	Normal	Normal
Abdomen / Tympanic	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Umbilicus:	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Slightly Painful, swollen. Hernia.	Slightly swollen. Hernia	Normal Hernia	Normal Hernia.	Normal. Hernia.
Articulations:	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Feces:	Watery / Fluid, yellowish.	Mushy, brownish –yellow	Pasty, brownish – yellow	Watery, brownish – yellow	Watery, yellowish grey	Thin mushy, brownish yellow	Mushy yellowish	Thin mushy, yellowish.	Thin mushy, yellow	Thin mushy, yellow	Mushy, brownish – yellow. Gelatinous	Mushy yellow (normal)
Diarrhea:	Yes	No	No	Yes	Yes	Yes	No	Yes	Yes	Yes	No	No

Attached Table no. 5: General examination of the calf no. 1 that excreted *Cryptosporidium* spp. oocysts.

Note: General appearance was good, except on days 7 and 8 where the calf was weak and had difficulties in standing.

Patient No. 2: Female, Holstein black and white calf. 5 – 6 days old.

Day / Parameter	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Temperature (38,5–39,5ºC)	39,5	38,3	38,5	39,0	39,0	39,8	38,3	39,2	39,4	39,0	38,6	39,0	39,0	38,7	38,5	39,0
Heart	Normal	Normal	Normal	Normal			Normal	Normal		Normal			Normal			
Hydration status	Normal	Normal	Normal	Normal			Normal	Normal		Normal			Normal			
Mucous membranes	Normal	Normal	Normal	Normal			Pale	Normal.		Normal			Normal			
Lung sounds:	Normal	Slightly increased	Moderate increase	Moderate increase. Coughing.			Moderate increase	Slightly increased		Slightly increased			Slightly increased. Coughing.			
Abdomen / Tympanic	Negative	Negative	Negative	Negative			Negative	Negative		Negative			Negative			
Umbilicus:	Normal	Normal	Normal	Normal			Normal	Normal		Normal.			Normal			
Articulations:	Normal	Normal	Normal	Normal			Normal	Normal		Normal			Normal			
Feces:	Thin mushy, yellowish.	Mushy, yellow	Watery, yellow. With fibrin.	Thin mushy, brownish – yellow			Mushy, yellowish brown	Pasty, yellow brownish		Mushy, yellow			Mushy yellow			
Diarrhea:	Yes	No	Yes	Yes			No	No		No			No	No	No	No
Others:			Purulent nasal discharge.			Purulent nasal discharge, inflammation around the ear tags	Purulent inflammation of the ears	Purulent inflammation of the ears.		Purulent inflammation of the ears			Mucous nasal discharge. On day 13: Purulent infl. of the ears.			

Attached table no. 6: General examination of the calf no. 2 that excreted *Cryptosporidium* spp. oocysts.

Note: General appearance was good, except on day 7, 8 and 9, where the calf was weak and had difficulties to stand.

Patient No. 3:

Female, Simmental, 6 days of age.

Day / Parameter	1	2	3	4	5	6	7	8	9
Temperature (38,5 – 39,5º C)	39,2	39,6		39,5	39,4	40,4	39,7	38,6	38,1
Heart	Normal	Normal		Normal	Normal	Normal	Normal		
Hydration status	Highly dehydrated	Moderately dehydrated		Moderately dehydrated	Slightly dehydrated	Normal	Normal		
Mucous membranes	Pale	Normal		Normal	Normal	Normal	Normal	Pale	Normal.
Lung sounds:	Increased. Abdominal breathing.	Slightly increased		Moderate increase	Moderate increase	Slightly increased	Moderate increase		
Abdomen:	Negative	Negative		Negative	Negative	Negative	Negative		
Umbilicus:	Normal	Normal		Normal	Normal	Normal	Normal		
Articulations:	Normal	Normal		Normal	Normal	Normal	Normal		
Feces:	Watery, yellowish. Traces of blood.	Watery, yellowish. Traces of blood.		Watery, greenish.	Thin mushy, yellowish brown. With Mucus and bad smell	Thin mushy, brownish yellow	Mushy, yellowish		
Diarrhea:	Yes	Yes		Yes	Yes	Yes	No		
Others:					Small round lesion on muzzle.	Swelling on infusion site. Fur loss on hip joints.	Swelling on infusion site. Fur loss on hip joints. Lesion on muzzle and gengiva.		

Attached table no. 7: General examination of the calf no. 3 that excreted *Cryptosporidium* spp. oocysts.

Note: General appearance: Weakness and inability to stand in the first two days. Moderate health condition with difficulties to stand up until day 8. On the last day the condition was good and the animal could stand up alone.

Patient no. 4: Female, Galloway. 6 days of age.

Day / Parameter	1	2	3	4	5	6	7
Temperature (38,5 – 39,5º C)	37,8	38,0	39,1		38,7	38,8	38,4
Heart	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Hydration status	Modarately dehydrated	Normal	Normal	Normal	Normal	Normal	Normal
Mucous membranes	Normal	Reddened	Reddened	Normal	Normal	Light red	
Lung sounds	Normal	Normal	Slightly increased	Normal	Normal	Normal	Normal
Abdomen /tymp	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Umbilicus:	Normal	Slightly painful	Normal	Normal	Normal	Normal	Normal
Articulations:	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Feces:	Watery greenish yellow. With fibrin.	Watery greenish yellow. With mucus.	Watery, yellow. With fibrin.	Watery, yellow. With fibrin.	Watery, yellow. With fibrin.	Mushy, brownish yellow. Mucus and traces of blood.	Pasty, yellowish
Diarrhea:	Yes	Yes	Yes	Yes	Yes	No	No

Attached table no. 8: General examination of the calf no. 4, that excreted *Cryptosporidium* spp. oocysts.

Note: General appearance was good on the first day with only some reduction of the suckle reflex. Only on days 2 and 3 the calf was weak, in a poor general state and unable to stand up alone. On the other days the general appearance was good.

Day / Parameter	8	9	10	11	12	13	14	15	16	17	18	19
Temperature (38,5 – 39,5ºC)	39,4	38,7	38,7	38,6	38,6	38,8	38,2	38,7	37,9	38,7	39,1	39,2
Heart	Normal	Normal		Normal	Normal		Normal	Normal		Normal	Normal	
Hydration status	Slight dehydration	Slight dehydration		Normal	Normal		Normal	Normal		Normal	Normal	
Mucous membranes	Pale	Pale		Slightly red	Normal		Slightly red	Normal		Reddened	Reddened	Normal
Lung sounds	Normal	Slightly increased		Slightly increased	Slightly increased		Normal	Slightly increased		Slightly increased	Normal	
Abdomen / Tymp.	Negative	Negative		Negative	Negative		Negative	Negative		Negative	Negative	
Umbilicus:	Normal. Slightly swollen and firm.	Normal.		Normal	Normal		Normal	Normal Slightly firm	painful and swollen	slightly firm and painful.	Normal	
Articulations:	Normal	Normal		Normal	Normal		Normal	Normal		Swollen carpal joints	Normal	
Feces:	Watery, greenish yellow	Thin mushy, green yellowish brown		Pasty, brownish – yellow	Pasty to mushy, yellowish brown		Mushy, yellowish brown	Mushy, yellowish brown		Mushy-pasty, brownish yellow	Pasty/mushy, brownish – yellow	
Diarrhea:	Yes	Yes		No	No		No	No		No	No	
Others:	Thromboflebitis on the left jugular vein.				Mild conjunctivitis on left eye					Thromboflebitis on left neck. Bruxism.	Thromboflebitis on left side of the neck. Bruxism.	

Attached table no. 9: General examination of the calf no. 4, that excreted *Cryptosporidium* spp. oocysts, during its second stay at the clinic.

Note: General appearance was moderate to good. On days 10, 15 and 16 the appearance was reduced, the calf was weak and had difficulties in standing up.

Patient no. 5: Red-Holstein, male. 14 days old.

Days / Parameter	1	2	3	4	5
Temperature (38,5–39,5ºC)	39,1	38,9	39,2	39,5	38,8
Heart	Normal	Normal	Normal		Normal
Hydration status	Slightly dehydrated	Slightly dehydrated	Normal		Slightly dehydrated
Mucous membranes	Normal	Normal	Normal		Normal
Lung sounds	Slightly increased	Normal	Normal		Normal
Abdomen / Tymp.	Negative	Negative	Negative		Negative
Umbilicus:	Normal	Normal	Normal		Normal
Articulations:	Normal	Normal	Normal		Normal
Feces:	Watery, yellowish green	Watery, yellowish green	Watery, brownish		Mushy, brownish
Diarrhea:	Yes	Yes	Yes		No

Attached table no. 10: General examination of the calf no. 5, that excreted *Cryptosporidium* spp. oocysts. Note: General appearance was moderate to good.

Patient no. 6: Red-Holstein, male. 7 days of age.

Day / Parameter	1	2	3	4	5
Temperature (38,5– 39,5ºC)	39,8	38,6	39,0	39,7	38,8
Heart	Normal	Normal	Normal		Normal
Hydration status	Slightly dehydrated	Normal	Normal		Normal
Mucous membranes	Normal	Normal	Normal		Normal
Lung sounds	Normal	Normal	Normal		Normal
Abdomen / Tymp.	Negative	Negative	Negative		Negative
Umbilicus:	Normal	Normal	Normal		Normal
Articulations:	Normal	Left carpus swollen. Rest normal.	Normal		Normal
Feces:	Thin mushy yellowish	Pasty to mushy, yellowish brown	Mushy, yellowish		Pasty, yellowish
Diarrhea:	Yes	No	No		No

Attached table no. 11: General examination of the calf no. 6, that excreted *Cryptosporidium* spp. oocysts. Note: General appearance was moderate to good on the 5 days the calf staved in the clinic.

Patient no. 7: Black Holstein Black and white, female. 8 days of age.

Day / Parameter	1	2	3	4	5	6	7	8	9	10	11	12
Temperature (38,5– 39,5ºC)	37,9	39,0	39,4	39,0	39,1	38,6	38,8	38,8	38,6	38,8	39,1	38,6
Heart	Normal	Normal	Normal	Normal						Normal		
Hydration status	Slightly dehydrated	Normal	Normal	Normal						Normal		
Mucous membranes	Pale	Normal	Normal	Pale						Normal		
Lung sounds	Slightly increased	Normal	Normal	Normal						Normal		
Abdomen / Tymp.	Negative	Negative	Negative	Negative						Negative		
Umbilicus:	Normal	Normal	Normal	Normal						Normal		
Articulations:	Normal	Normal	Normal	Normal						Normal		
Feces:	Thin mushy to watery, greenish yellow	Thin-mushy to watery, yellowish brown	Pasty, yellowish	Mushy, yellowish brown						Mushy, yellowish brown		
Diarrhea:	Yes	Yes	No	No						No		
Others:												

Attached table no. 12: General examination of the calf no. 7, that excreted *Cryptosporidium* spp. oocysts.

Note: General appearance was moderate in the first day, changing to good in the remaining stay in the clinic.

Patient no. 8: Black and white Holstein, male. 12 days of age.

Day / Parameter	1	2	3	4	5	6	7
Temperature (38,5– 39,5ºC)	37,8	39,5	39,8	39,0	38,8	38,8	38,6
Heart	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Hydration status	Slightly dehydrated	Slightly dehydrated	Slightly dehydrated	Normal	Normal	Normal	Normal
Mucous membranes	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Lung sounds	Normal	Slightly increased	Normal	Normal	Normal	Normal	Normal
Abdomen / Tymp.	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Umbilicus:	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Articulations:	Normal	Normal	Normal	Normal	Normal	Tarsal joints painful	Tarsal joints painful
Feces:	Watery to thin mushy yellowish	Thin mushy yellowish	Watery to thin mushy yellowish	Thin mushy to mushy brownish yellow	Thin mushy, brownish yellow	Thin mushy yellowish	Mushy, yellowish
Diarrhea:	Yes	Yes	Yes	No	Yes	Yes	No
Others:	Mucous nasal discharge			Mucopurulent nasal discharge			

Attached table no. 13: General examination of the calf no. 8, that excreted *Cryptosporidium* spp. oocysts.